REMARKS

STATUS OF THE CLAIMS:

Claims 1 to 20, 26 to 30, 32, 36, 37, and 38 to 40 are cancelled.

Claim 21 was amended.

New Claim 45 was added.

Claims 21 to 25, 31, 33 to 35, and 41 to 45 are pending.

Claim 21 was amended to delete sub-clause "(c)" in its entirety, and to append the term "and" after sub-clause "(a)" to place this claim in proper Markush format in consideration of the deletion of sub-clause "(c)". Applicants assert that this amendment was not made to overcome any issues related to the patentability of this claim, but rather to place this claim and its dependent claims in better condition for allowance. Applicants reserve the right to prosecute Claim 21 as originally presented in related applications. Applicants right to equivalents of Claim 21 is reserved. No new matter has been added.

Support for new Claim 45 may be found in Claim 21 as originally presented.

I. **Rejections under 35 U.S.C. § 101**

a. The Examiner has rejected Claims 21 to 25, 31, 33 to 35, and 41 to 44 under 35 U.S.C: § 101, for failure to demonstrate a specific and substantial asserted utility or a well-established utility. More particularly, the Examiner alleges that "First, as was stated in the previous Office Action, the specification disclosed a novel nucleic acid molecules of SEQ ID NO: 1 encoding SH2/SH3 domain --containing protein h SLAP-2 of SEQ ID NO:2. The specification fails to provide sufficient objective evidence of any activity for encoded protein. Applicant only states that said protein shows 47 % identity to human SLAP and 58 % identity to the mouse SLAP proteins (see Table 4 and page 61, lines 22-30 in particular). The specification disclosed that based on sequence homology to related molecules, said protein may be a novel human SLAP-2 protein. The specification also disclosed that said hSLAP-2 nucleic acid sequence and related protein can be used for diagnosing, treating or preventing disorders or diseases associated with aberrant or uncontrolled cellular signal transduction or with hyperactive cell, or may play a role in one or more aspects of regulating the immune system and tumor cell biology (see page 20, lines 5-20 and page 41, lines 22-30 in particular). No well-established utility for a human SLAP-2 protein is indicated. Moreover, in addition to previously cited references indicating that homology -based prediction of protein function is unreliable, newly cited references of Whisstock et al., (Quarterly Review of Biophysics, 2003, 36, pp307-340) teaches that prediction of protein function from sequence and structure is difficult problem, because homologous proteins often have different function. A fundamental problem is that function is in many cases an ill-defined concept (see Abstract in particular). Even in situations where there is some confidence of a similar overall structure between two proteins, only experimental research can confirm the artisan's best guess as to the function of the structurally related protein (see in particular "Abstract" and Box 2). Thus, in light of the art recognized fact that minor sequence differences can significantly affect a protein's function, one skilled in the art would find it more likely than not that h SLAP-2 of SEQ ID NO:2 is not having the same function as human SLAP. The recitation of percent identity language, in the absence of a *testable function* and limitations regarding the *sequence length over which the percent identity is required* does not allow the Skilled artisan reasonable believed that hSLAP-2 is a new member of the SLAP family of adapter proteins. Thus, the homology-based assignment h SLAP-2 of SEQ ID NQ:2 as human SLAP receptor does not appear to provide evidence of a specific and substantial utility based on the knowledge of the skilled artisan and the data presented in the instant specification.".

Applicants disagree and point out that the Examiner's position on utility is not in accordance with US patent law, nor is the Examiner's position in accordance with the guidance provided by the U.S.P.T.O in the Revised Interim Utility Guidelines. As Applicants pointed out in their August 18th, 2003 Reply and in their May 3rd, 2004 Reply, U.S. patent law does not require that a specification actually demonstrate use of a claimed invention. Rather, it is established law that a disclosure is enabling so long as it contains information which would lead one of ordinary skill in the art to *reasonably believe* the claimed invention has utility. *In re Barr*, 170 U.S.P.Q. 330 (C.C.P.A. 1971).

In the absence of evidence or apparent reason why the claimed polynucleotides do not possess the disclosed utility, the allegation of utility in the specification *must* be accepted as correct. *Ex parte Krenzer*, 199 U.S.P.Q. 227 (Pat. Off. Bd. App. 1978).

Applicants also point out that the patent laws do not require a direct case-and-effect relationship between a gene and a disease in order to establish a specific, substantial, and credible utility. Rather, only a "reasonable correlation" is required (Cross v. Iizuka, 753 F.2d 1040, 224 USPQ 739 (Fed. Cir. 1985); In re Jolles, 628 F.2d 1322, 206 USPQ 885 (CCPA 1980); Nelson v. Bowler, 626 F.2d 853, 206 USPQ 881 (CCPA 1980)). In addition, such a "reasonable correlation" need not rise to the level of statistical certainty (Nelson v. Bowler, 626 F.2d 853, 857, 206 USPQ 881, 884 (CCPA 1980)), nor does the correlation need to be "rigorous" (see Fujikawa v. Wattanasin, 93 F.3d 1559, 1565, 39 USPQ2d 1895, 1900 (Fed. Cir. 1996)).

Applicants pointed out to the Examiner in Applicants August 18th, 2003 Reply and in their May 3rd, 2004 Reply the objective evidence disclosed in Applicants specification that support Applicants assertion that one skilled in the art would reasonably believe that hSLAP-2 is a new member of the SLAP family of adapter proteins based not only on the high percent identity shared between the human and mouse SLAP proteins, but also the presence of the conserved SH2/SH3 domains which are essential to adaptor protein function (see pages 10 to 14 of Applicants August 18th, 2003 Reply). Applicants believe this information is sufficient to establish that a skilled artisan would reasonably believe that hSLAP-2 is a new member of the SLAP family of adapter proteins.

Applicants also pointed out to the Examiner further objective evidence disclosed in Applicants specification supporting Applicants assertions that one skilled in the art would reasonably believe that hSLAP-2 is a new member of the SLAP family of adapter proteins based upon the high percent identity between the hSLAP-2 SH2 and SH2/SH3 domains themselves to the SH2 and SH2/SH3 domains of Lyn and Hck tyrosine kinases from the Src-family. Applicants pointed out the significance of this result by referring the Examiner to the teachings of Kelly et al., Curr. Opin. Immunol., 12:267-275 (2000); Tomlinson et al., Immunol. Today 21:584-591 (2000); Myung et al., Curr. Opin. Immunol., 12:256-266 (2000); and Kurosaki, T. et al., Ann. Rev. Immunol., 17:555-592 (1999), which, in summary, provide the basis for the appreciation in the art that distinct signaling cascades required for lymphocyte activation depend upon the involvement of specific adaptor proteins.

Applicants also pointed out to the Examiner that the hSLAP-2, like SLAP, has a restricted expression pattern being primarily expressed in "immune system cells includ[ed]ing peripheral

blood lymphocytes, Jurkat T-cells and bone-marrow cells". In consideration of the totality of evidence provided in Applicants specification, Applicants asserted that one skilled in the art would reasonably believe that hSLAP-2 is a new member of the SLAP family of adapter proteins.

After establishing the strong supporting evidence that hSLAP-2 is a new member of the SLAP family of adapter proteins, Applicants also pointed out the utilities for hSLAP-2 as disclosed in Applicants specification. Specifically, Applicants pointed out that the specification teaches that the hSLAP-2 polypeptide is an adaptor protein which functions "in the receptor-ligand signal transduction pathway in cells of the hematopoietic lineage" (see paragraph 54 of specification). More particularly, Applicants specification teaches that hSLAP-2 is a "negative regulator[s] of intracellular signal transduction in several cell types, including T-cells" (see paragraph 76). Applicants specification also teaches that hSLAP-2 is useful for "the diagnosis, screening, monitoring, therapy, and prevention of immune system related conditions or diseases, particularly those involving T-cell and B-cell neoplasms; inflammation disorders, diseases and conditions, rheumatoid arthritis, osteoarthritis, psoriasis, rhinitis, inflammatory bowel disease (Crohn's and ulcerative colitis), allergies, particularly those involving hyperactivity of B-cells and T-cells, or other immune cells, such as mast cells or eosinophils; autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis; pulmonary diseases including asthma, acute respiratory distress syndrome, and chronic obstructive pulmonary disorder; tissue/ organ rejection; and cancer" (see paragraph 12).

Applicants also referred the Examiner to three post-filing publications from three independent groups, namely Pandey et al, Holland et al, and Loreto et al, that completely corroborated the teachings of Applicants specification relating to the description of hSLAP-2 as a new member of the SLAP family of adaptor proteins, in addition to its utility as a negative modulator of T-cell activation. Specifically, Pandey et al, Holland et al, and Loreto et al identified a protein identical to hSLAP-2, recognized hSLAP-2 as representing a new member of the SLAP family of adapter proteins using the same criteria utilized by Applicants (e.g., percent homology, shared structural features, etc.), and established experimentally that hSLAP-2 functions as originally conceived in Applicants specification (e.g., as a negative modulator of T-cell activation).

Applicants also pointed out to the Examiner that the instant specification teaches that hSLAP-2 is capable of binding to ZAP-70 and that one skilled in the art would appreciate that any molecule that binds to ZAP-70 would be expected to affect T-cell receptor signaling and thus would be useful as a target for therapeutic intervention for disorders affecting T-cell antigen receptor

signaling, such as T-cell tumors, lymphomas, leukemias, thymomas, and autoimmune disorders, among others (see paragraph 9). This rational is supported by the teachings of Chen et al., Cell 71:649-662 (1992); Zhang et al., Cell 92:83-92 (1998); Chan et al., EMBO J. 14:2499-2508 (1995); Williams et al., J. Biol. Chem. 271:19641-19644 (1996); and Williams et al., Mol. Cell. Biol., 18:1388-1399 (1998); and is based upon the fact that ZAP-70 links the activated T-cell receptor to downstream signaling events that ultimately leads to the transcription of genes such as IL-2, which is a hallmark of T-cell activation.

Both Pandey et al and Loreto et al demonstrate experimentally that hSLAP-2 is capable of binding to ZAP-70 and teach that this is the mechanism by which hSLAP-2 negatively affects T-cell receptor activation. Applicants believe this information alone demonstrates that hSLAP-2 has a well-established utility and was specifically taught by the teachings of Applicants specification.

In addition, Applicants also point out that since hSLAP-2 has been shown to negatively regulate T-cell receptor (TCR) signaling, it automatically implicates hSLAP-2 as functioning in the co-stimulatory pathway. As the Examiner will appreciate, in order for T-cells to be fully activated they require 2 signals, the first from the T-cell receptor itself, and the second from a costimulatory signal such as CD28 or CTLA-4. This costimulatory signal provides an amplification of the TCR signal which enables the T-cell to proliferate ultimately leading to the production of cytokines including IL-2, as described *supra*. In addition to the positive costimulatory signal delivered by CD28, a negative signal is provided via CTLA-4 which inhibits T-cell activation, proliferation and cytokine production. The balance between CD28 and CTLA-4 expression determines whether the T-cell will be activated or not.

In cancer patients, or animals with tumors, it is known in the art that the host immune system is suppressed and T-cell responses are diminished thus enabling the cancer to evade recognition by the immune system and continue to proliferate. Therefore, one well-established approach to treating cancer is disruption of the co-stimulatory pathway – an approach known as immunomodulatory therapy. Leach et al (Science, 22;271(5256):1734-6 (1996); submitted concurrently herewith for the convenience of the Examiner) describe an antibody directed against CTLA-4 which has been used to activate the immune system. These types of antibodies block CTLA-4 function, thereby, stimulating T-cell proliferation and function. Studies from the Leach et al group have shown that an anti-CTLA-4 antibody has anti-tumor activity in animals and clinical studies are ongoing with similar types of molecules. Therefore, by analogy, since hSLAP-2 acts to attenuate immune responses, an antagonist directed against hSLAP-2 would similarly be expected to

have anti-tumor activity largely due to disruption of ZAP-70 binding and thus amplification of T-cell receptor activation.

Disruption of the co-stimulatory pathway is also a well-known mechanism for treating various immune disorders. CTLA4-Ig is an Ig fusion protein that has been shown to potently inhibit T-cell proliferation in vitro, like hSLAP-2 (see Tan et al, J. Exp. Med., 177:165-173 (1993); submitted concurrently herewith for the convenience of the Examiner), in vivo (see Webb et al., Eur. J. Immunol., 26:2320-2328 (1996); and Finck et al., Science, 265:1225-1227 (1994); submitted concurrently herewith for the convenience of the Examiner), and in human subjects (see Kremer et al., NEJM, 349(20); 1907-1915 (2005); submitted concurrently herewith for the convenience of the Examiner) and is currently in advanced clinical trials for the treatment of rheumatoid arthritis. Clearly, one skilled in the art would recognize that hSLAP-2, a protein that affects the same co-stimulatory pathway, would reasonably be expected to have the same utility as CTLA4-Ig.

Although Applicants firmly believe that the Examiners utility rejection has been overcome, Applicants would also like to refer the Examiner to the further teachings of McGlade et al (International Publication No. WO 02/42452, published May 30th, 2002; originally submitted with Applicants June 2nd, 2003 IDS). The teachings of McGlade et al are consistent with the teachings of Applicants specification in addition to the teachings of Pandey et al, Holland et al, and Loreto et al, and provide further post-filing publication corroborative evidence that the hSLAP-2 has utility, is in fact a new member of the SLAP family of adaptor proteins, and functions as a negative regulator of T-cell receptor activation. McGlade describes results obtained for a molecule that is 100% identical to hSLAP-2 which they refer to as MARS. McGlade teaches that hSLAP-2 inhibits T-cell receptor mediated NFAT activation (see page 35) which is consistent with its utility as a negative regulator of T-cell receptor activation since NFAT is a transcription factor that is activated by the T-cell receptor and results in transcriptional upregulation and expression of IL-2. McGlade further teaches that hSLAP-2 maps to chromosome 20 in a region that is frequently deleted in myeloproliferative disorders (see page 44), and in particular, premalignant hyperproliferative disorders of the myeloid cell population. McGlade also demonstrated that a cohort of patients with monoallelic deletions of chromosome 20q11 were found to have the hSLAP-2 specifically deleted. The latter finding directly associates the deletion of hSLAP-2 to the incidence of premalignant hyperproliferative disorders of the myeloid cell population. Applicants point out that the latter finding is directly corroborative with the teachings of Applicants specification relative to the utility of hSLAP-2 (see arguments presented *supra*, the arguments presented in Applicants August 18th, 2003 Reply, in addition to the utilities

asserted in paragraphs 9, 12, 54, and 76 of Applicants specification). Applicants adamantly assert that hSLAP-2 adequately satisfies all tenets of the utility requirement and request that the utility rejection be withdrawn.

In consideration of the fact that 1.) the requisite teachings demonstrating that hSLAP-2 is a new member of the SLAP family of adaptor proteins is found within Applicants specification as originally filed; 2.) the fact that the description of the anticipated function and utility of hSLAP-2 is found within Applicants specification as originally filed (e.g., "negative regulator[s] of intracellular signal transduction in several cell types, including T-cells"); 3.) the fact that Applicants specification described one of the ligands for hSLAP-2 (e.g., ZAP-70); 4.) the fact that modulation of ZAP-70 is directly linked to the asserted negative T-cell receptor activation utility of hSLAP-2; 5.) the fact that T-cell receptor activation is controlled by the co-stimulatory pathway; 6.) the fact that disruption of the co-stimulatory pathway is a well-established mechanism for treating immune (e.g., Tan et al, Webb et al, Finck et al, Kremer et al, etc.) and oncology (e.g., Leach et al, etc.) diseases and disorders; 7.) the fact that negative regulation of T-cell receptor activation is known in the art to lead to T-cell tumors, lymphomas, leukemias, thyomas, and other proliferative conditions; and 9.) the fact that four independent groups, namely Pandey et al, Holland et al, Loreto et al, and McGlade et al, published papers confirming that hSLAP-2 is a new member of the SLAP family of adaptor proteins, has the same physiological function, and functions via the same mechanism as taught in Applicants specification as originally filed; supports Applicants arguments that hSLAP-2 has a specific, substantial, and credible or well-established utility and that the Examiners maintenance of the utility rejection for the pending claims is erroneous and should be withdrawn.

> **b.** The Examiner has rejected Claims 21 to 25, 31, 33 to 35, and 41 to 44 under 35 U.S.C. § 101, for failure to demonstrate a specific and substantial asserted utility or a well-established utility. More particularly, the Examiner alleges that "instant specification it is clearly stated that SLAP, not hSLAP-2, have been shown to be a negative regulator of intracellular signal transduction in several cell types including T cells".

Applicants do not refute that the specification makes this statement. However, the Examiner's basis for making this statement is unclear since it doesn't have any bearing on whether the instant claims have utility. Specifically, Applicants specification makes it quite clear, from both a structural and functional point of view, that hSLAP-2 is a member of the SLAP family of proteins and that one skilled in the art, upon reading the instant specification, would appreciate that hSLAP-2 would **also** be a "negative regulator of intracellular signal transduction in several cell types including

T cells" on account of it being a member of this select group. The latter is reinforced considering that hSLAP-2 was only the second member of this family to have been discovered at the time Applicants filed the instant application (with SLAP being the other member). On account of the arguments presented *supra*, in conjunction with knowledge in the art of the role SLAP plays as a negative regulator of T-cell activation, it is clear that an antagonist of hSLAP-2 would be therapeutically desirable. Since the teachings of Applicant's specification are completely consistent with antagonism of hSLAP-2, Applicants assert that the instant specification does disclose the utility of hSLAP-2 as being a negative regulator of T-cell activation.

First, Applicants specification teaches that hSLAP-2 is a member of the SLAP family stating that "the discovery of human SLAP-2, a new member of the SLAP family of adapter proteins" (see page 4, paragraph 0012); that the "gene and encoded product according to the present invention are called hSLAP-2 (human Src-Like Adapter Protein-2) due to its similarity with both human SLAP (hSLAP) and mouse SLAP (mSLAP) sequences." (see page 20, paragraph 0076); and that the "SLAP proteins have been shown to be negative regulators of intracellular signal transduction in several cell types, including T-cells (see: Roche, S. et al., (1998) Src-like adaptor protein (Slap) is a negative regulator of mitogenesis. *Curr. Biol.* 8:975-978; Tang, J. et al., (1999) SLAP, a dimeric adapter protein, plays a functional role in T cell receptor signaling. *Proc. Natl. Acad Sci. USA* 96:9775-9780; and Sosinowski, T. et al., (2000) Src-like adaptor protein (SLAP) is a negative regulator of T cell receptor signaling. *J. Exp. Med.* 191:463-474)." (see page 20, paragraph 0076).

Applicants specification clearly teaches that hSLAP-2 is a member of the SLAP family of proteins. However, the Examiner appears to allege that this has not been demonstrated since "homology-based prediction of protein function is unreliable". As Applicants noted above, such statements do not apply to the instant invention due to the fact that exceptions to this very general rule do exist as corroborated by the teachings of Pandey et al, Holland et al, Loreto et al, and McGlade et al. Additionally, the Examiner takes this allegation one step further by stating "the original members of the [SLAP] family were not classified based on their biological activity, but rather, by their common structure and the fact that they are adaptor proteins. Without some common biological activity for the family members, a new member would not have a specific, substantial, or credible utility when relying only on the fact that it has structural similarity to the other family members and is also a adaptor protein." Applicants do not refute that adaptor proteins, in general, have divergent functions. However, the entire field of adaptor protein biology is not at issue before the Examiner. Rather, since hSLAP-2 is alleged to be a member of the "SLAP-family of adaptor

proteins", the only issue is whether the specification teaches that hSLAP-2 has the same function and hence the same utility as SLAP (i.e., as a "negative regulator of T-cell activation").

The teachings of Applicants specification, as originally filed, is completely consistent with Applicants assertion that hSLAP-2, being a member of the SLAP family, is also useful as a "negative regulator of T-cell activation".

First, Applicants point out that the instant specification explicitly states that "hSLAP-2 protein and encoding nucleic acid can be used as effectors in methods to affect T-cell activation". According to the Random House Webster's Unabriged Dictionary, 2nd Edition, New York, 1998, "affect" is defined as "1. to act on; produce an effect or change in"; and "3. (of pain, disease, etc.) to attack or lay hold of". In addition, Applicants point out that the usage of this term is typically in a "negative" context, so as to "inhibit", which is supported by the definition of this terms adjective "affected": "2. influenced in a harmful way; impaired, harmed, or attacked…" (ibid).

"The hSLAP-2 protein and nucleic acid can be used in screening assays of candidate bioactive agents that modulate hSLAP-2 bioactivity, for potential use to treat T- and B-cell disorders, such as tumors, lymphomas, and leukemias, or to treat inflammation disorders, such as those involving T-cells. In addition, **hSLAP-2 protein and encoding nucleic acid can be used as effectors in methods to affect T-cell activation**. By "modulate" herein is meant that the bioactivity of hSLAP-2 is altered, i.e., either increased or decreased. In a preferred embodiment, hSLAP-2 bioactivity is inhibited. hSLAP-2 is a member of the class of adapter proteins involved in T-cell activation and T-cell responses; thus, it may play a role in antigen-presenting cells such as B-cells. Accordingly, hSLAP-2 can be used as a target to screen for inhibitors of its function or expression. " (p. 44, paragraph 0147)

Clearly, one skilled in the art would recognize that Applicants specification teaches that hSLAP-2 is also useful as a "negative regulator of T-cell activation" by this statement alone, in addition to the supporting statements in the specification as originally filed as described *infra* and *supra*.

Second, Applicants specification teaches that an "antagonist" of hSLAP-2 would be desirable, which based knowledge of the co-stimulatory pathway in the art, is precisely what a skilled artisan would expect the specification to teach in support of hSLAP-2 being a "negative regulator of T-cell activation": (NOTE: EMPHASIS INTENTIONALLY ADDED)

"It is also an object of the present invention to provide a substantially purified **antagonist or inhibitor** of the polypeptide of SEQ ID NO:2. In this regard, and by way of example, a purified antibody that binds to a polypeptide comprising the amino acid sequence of SEQ ID NO:2 is provided." (p.6, paragraph 0021)

"The present invention encompasses the nucleic acid sequence (SEQ ID NO:1) encoding the full-length hSLAP-2 polypeptide (SEQ ID NO:2) and the use of compositions comprising the hSLAP-2 polynucleotide or polypeptide in methods for screening for **antagonists or inhibitors of the interaction of hSLAP-2 with cellular signaling components**. (p. 21, paragraph 0077)

"In another embodiment of the present invention, natural, modified, or recombinant nucleic acid sequences, or a fragment thereof, encoding hSLAP-2 polypeptide may be ligated to a heterologous sequence to encode a fusion protein. For example, for **screening peptide libraries for inhibitors or modulators of hSLAP-2 activity or binding**, it may be useful to encode a chimeric hSLAP-2 protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the hSLAP-2 protein-encoding sequence and the heterologous protein sequence, so that the hSLAP-2 protein may be cleaved and purified away from the heterologous moiety." (P. 27, paragraph 96)

"**Antagonists or inhibitors of the hSLAP-2 polypeptide of the present invention may be produced** using methods which are generally known in the art. In particular, purified hSLAP-2 protein, or fragments thereof, can be used to produce antibodies, or to screen libraries of pharmaceutical agents or other compounds, particularly, small molecules, synthetic or naturally occurring, to identify those which specifically bind hSLAP-2. (e.g. Libraries are commercially available from Sigma or Aldrich)." (p. 38, paragraph 0124)

"The transgenic animals, particularly transgenic mice, containing a nucleic acid molecule which encodes human hSLAP-2 may be used as animal models for studying *in vivo* the overexpression of hSLAP-2 and **for use in drug evaluation and discovery efforts to find compounds effective to inhibit or modulate the activity of hSLAP-2**" (p. 61, paragraph 0201).

Applicants also point out to the Examiner that some of the original claims were directed to antagonists of hSLAP-2, and not to agonists. See, for example, original Claim 16. In conjunction with the above references from the instant specification, it is clear that Applicants specification teaches that hSLAP-2 was believed to be a "negative regulator of T-cell activation" by the inventors

at the time the instant specification was originally filed and that the teachings of the specification are reflected in the same.

Applicants also point out additional statements that support Applicants assertions that the specification teaches that hSLAP-2 is a "negative regulator of T-cell activation":

"The hSLAP-2 protein and nucleic acid can be used in screening assays of candidate bioactive agents that modulate hSLAP-2 bioactivity, for potential use to treat T- and B-cell disorders, such as tumors, lymphomas, and leukemias, or to treat inflammation disorders, such as those involving T-cells. In addition, hSLAP-2 protein and encoding nucleic acid can be used as effectors in methods to affect T-cell activation. By "modulate" herein is meant that the bioactivity of hSLAP-2 is altered, i.e., either increased or decreased. **In a preferred embodiment, hSLAP-2 bioactivity is inhibited. hSLAP-2 is a member of the class of adapter proteins involved in T-cell activation and T-cell responses...Accordingly, hSLAP-2 can be used as a target to screen for inhibitors of its function or expression.** " (p. 44, paragraph 0147)

In addition, Applicants assertions that the instant specification teaches that hSLAP-2 is useful as a "negative regulator of T-cell activation", is further supported by the teachings describing the intent to identify hSLAP-2 antagonists that inhibit binding between hSLAP-2 and ZAP-70 since the role of hSLAP-2 in negatively regulating T-cell activation is directly due to its binding to ZAP-70 as supported by the teachings of Pandey et al and Loreto et al:

"**Inhibitors of human hSLAP-2** may be identified by screening compounds to ascertain their effect on hSLAP-2 activity. As described herein, in some embodiments of the present invention, **compounds are screened to identify inhibitors by contacting human hSLAP-2 with a molecule with which it binds or associates, (e.g., possibly ZAP-70, Syk, and LAT as suggested by published data with the SLAP protein; Tang, J. et al. (1999) *Proc. Natl. Acad Sci. USA* 96:9775-9780)**, in the presence or absence of a test compound. **Under conditions of the assay, the inhibitors will prevent or reduce binding of human hSLAP-2 to ZAP-70, for example. Antibodies which inhibit hSLAP-2/ ZAP-70 binding are useful as inhibitors and, therefore as positive controls in the assay**." (p.44, paragraph 0148)

"In a related embodiment, the **methods comprise screening for a bioactive agent capable of inhibiting the bioactivity of a hSLAP-2 protein. By "bioactivity" herein is meant the binding of the hSLAP-2 to any of its targets, for example, including ZAP-70, Syk, and LAT, as suggested by published data with SLAP protein. Thus, bioactive agents that prevent hSLAP-2**

binding, i.e., interrupt or block or inhibit the interaction of hSLAP-2 and its target molecule, may be found. The method comprises combining the hSLAP-2 protein and a candidate bioactive agent, and determining the binding of the candidate agent to hSLAP-2 protein." (p. 46, paragraph 0152)

Clearly, one skilled in the art would recognize that Applicants specification teaches that hSLAP-2 is also useful as a "negative regulator of T-cell activation" by these teachings alone since antagonizing the interaction between ZAP-70 and hSLAP-2 would be desirable only if hSLAP-2 was a "negative regulator of T-cell activation".

Applicants believe the Examiners statement that in "the instant specification it is clearly stated that SLAP, not hSLAP-2, have been shown to be a negative regulator of intracellular signal transduction in several cell types including T cells" has no bearing on whether the instant claims meet the utility requirement since the teachings of the instant specification are completely consistent with, and based upon, hSLAP-2 being a "negative regulator of T-cell activation".

> **c.** The Examiner has rejected Claims 21 to 25, 31, 33 to 35, and 41 to 44 under 35 U.S.C. § 101, for failure to demonstrate a specific and substantial asserted utility or a well-established utility".

The Examiner has consistently alleged that the utility of a protein cannot be demonstrated by showing that a protein is homologous to another protein that has a well-known utility. Applicants point out that this argument is clearly erroneous in consideration of the fact that Applicants correctly identified the sub-family of adaptor proteins to which hSLAP-2 belongs, correctly identified the physiological function of the hSLAP-2 protein, and correctly identified the mechanism by which hSLAP-2 mediates its function, as taught by Applicants specification as originally filed. Moreover, the fact that Pandey et al, Holland et al, Loreto et al, and McGlade et al all ascribe the same family and functional assignments to hSLAP-2 using the same homology criteria as Applicants also clearly demonstrates that this type of association is well-established and commonly accepted in the art. In consideration of the latter, Applicants request that the Examiner concede the same and to introduce corrective statements into the record. The Examiners utility rejection as it relates to homology determinations is moot and is no longer applicable to the pending claims.

Applicants do not refute the teachings of Whisstock et al, however Applicants assert that the Examiner's allegation that "prediction of protein function from sequence and structure is a difficult

problem" is moot considering exceptions to this difficulty clearly exist which as evidenced by the teachings of Applicants specification. The Examiner alleges that "[e]ven in situations where there is some confidence of a similar overall structure between two proteins, only experimental research can confirm the artisan's best guess as to the function of the structurally related protein". Applicants disagree and point to the teachings of Applicants specification again as evidence that experimental research is not required to accurately predict protein function. Nonetheless, the teachings of Pandey et al, Holland et al, Loreto et al, and McGlade et al do provide such experimental confirmation and thus the Examiner's allegation is moot.

Applicants disagree with the Examiner's allegation that "the specification does not disclose any diseases or conditions known to be associated with the hSLAP polypeptide, encoded by SEQ ID NO:2 or any conditions associated with altered levels (increase or decrease) of said polypeptide. Since any protein may potentially be used as a treatment agent, this utility would not be considered to be specific. Since no particular disease or condition is disclosed, the artisan would have been required to perform additional experimentation to identify and/or reasonably confirm the asserted use of hSLAP polypeptide as a treatment agent and therefore, this utility would not be considered to be substantial."

Specifically, Applicants pointed out that it is current U.S.P.T.O. policy, inclusive of the practice of Technology Group 1600, to recognize that a polynucleotide or polypeptide has utility based upon **either** its association to a disease or disorder, **or** a description of its physiological relevance. Applicants assert that while only satisfying one of these requirements is necessary, Applicants specification satisfies both of these requirements. First, as Applicants argued *supra*, the instant specification taught that the physiological function of hSLAP2 is to negatively regulate T-cell proliferation, and also taught that hSLAP-2 binds to ZAP-70, among others. One or both of the latter was directly corroborated by the teachings of Pandey et al, Holland et al, Loreto et al, and McGlade et al. Secondly, since this physiologic function is known in the art to be directly controlled by the co-stimulatory pathway, the disruption of which is currently the subject of several clinical investigations, is *prima facie* evidence that hSLAP-2 is associated with co-stimulatory diseases including cancer and immune disorders.

Moreover, Applicants pointed out to the Examiner that the asserted utilities are specific, substantial and that the skilled artisan would credibly believe that hSLAP-2 has the asserted utilities as a consequence of the strong supporting evidence that hSLAP-2 is a new member of the SLAP family of adapter proteins, and that it functions as a negative regulator of T-cell receptor activation.

Specifically, Applicants pointed out to the Examiner that the utility of hSLAP-2 is "specific" since modulation of T-cell receptor activation is specific to methods of treating and/or diagnosing immune disorders specific to aberrant T-cell receptor activity since "unregulated activation of the T-cell receptor (TCR) can lead to aberrant T-cell growth, resulting in, for example, T-cell tumors, lymphomas, leukemias and thymomas" (see Applicants August 18th, 2003 Reply, page 12). Since one of the utilities of hSLAP-2 relates to methods of treating aberrant T-cell receptor disorders, the Examiner is reminded that asserting the utility rejection on this basis is not in accordance with the guidance provided in the Revised Interim Utility Guidelines since "most diseases or conditions can be treated, rejections under 35 U.S.C 101 for treatment claims should rarely be made". Applicants request that the utility rejection be withdrawn in acknowledgement of this guidance.

Applicants also pointed out that the utility of hSLAP-2 represents a "substantial" utility and does not constitute a throw-away utility since its use in treating and/or diagnosing immune disorders specific to aberrant T-cell receptor activity represents a "real world" context of use. Since such a method of treatment necessarily encompasses specific diseases or disorders exemplified in Applicants specification, including for example, T-cell tumors, lymphomas, leukemias and thymomas, among others, Applicants point out that such a utility represents a "substantial" utility in accordance with the Revised Interim Utility Guidelines. The Guidelines that a method of treating a disorder represents a substantial utility unless the method does not specify the disease or condition to be treated. However, since Applicants specification describes specific diseases or disorders that hSLAP-2 would be useful in treating, the "substantial utility" criterion has been met. Applicants request that the utility rejection be withdrawn in acknowledgement of this guidance.

Applicants also pointed out that the utility of hSLAP-2 is "credible" since one skilled in the art would clearly appreciate that hSLAP-2 is a new member of the SLAP family of adaptor proteins and would be expected to have the asserted utilities based upon the teachings of Applicants specification. The issue of whether such asserted utitilties are "credible" is moot in consideration of the independent corroborating support provided by Pandey et al, Holland et al, and Loreto et al. Applicants again point the Examiner to the guidance provided by the Revised Interim Utility Guidelines which state that an "assertion is credible unless (A) the logic underlying the assertion is seriously flawed, or (B) the facts upon which the assertion is based are inconsistent with the logic underlying the assertion". Since the asserted utilities were independently corroborated by three separate post-filing publications, Applicants adamantly assert that the "logic underlying the assertion" is clearly not flawed, nor are the facts flawed, since each of these publications support the

teachings of Applicants application as originally filed relative to the hSLAP-2 representing a new member of the SLAP family of adaptors proteins, in addition to its role as a negative regulator of T-cell receptor activation. Applicants request that the utility rejection be withdrawn in acknowledgement of this guidance.

As Applicants stated in the August 18th, 2003 Reply, Applicants do not agree with the Examiners alleged application of Brenner v. Manson to the pending claims of the instant application. At issue in Brenner was whether a chemical process for synthesizing chemical compounds was patentable for an application that did not disclose any utility for the disclosed compounds (i.e., the patent application at issue in Brenner did not even describe the utility of the class of compounds that were orthologous to the claimed compounds at issue in the case either explicitly or through reference to a publication). Applicants assert that the instant patent application explicitly discloses the utility of the hSLAP-2 polynucleotide and polypeptides, in addition to any modulators thereof, as originally filed. Thus, since the utility of hSLAP-2 is already disclosed in the specification, Brenner v. Manson cannot apply.

II. Rejections under 35 U.S.C. § 112, first paragraph

a. The Examiner has rejected Claims 21 to 25, 31, 33 to 37, and 41 to 44 under 35 U.S.C. § 112, first paragraph, alleging that the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility and that one skilled in the art clearly would not know how to use the claimed invention.

Applicants disagree. Applicants believe the Examiners allegations have been overcome in light of the arguments presented above, the arguments presented in the Applicants August 18th, 2003 Reply, the teachings of Applicants specification, in addition to the subsequent corroborative teachings of Holland et al, Pandey et al, Loreto et al, and McGlade et al. Since hSLAP-2 has a specific, substantial, and well established utility in the specification as originally filed, one skilled in the art clearly would know how to use the claimed invention. In addition, Applicants also assert that since the hSLAP-2 sequence, function, as well as its biological significance are disclosed in the specification as originally filed, Applicants specification provides the requisite teachings that a skilled artisan would require to use the claimed invention. Applicants request that the rejection under U.S.C. § 112, first paragraph be withdrawn for Claims 21 to 25, 31, 33 to 35, and 41 to 44. The rejection of Claims 36 and 37 is moot in consideration of Applicants cancellation of Claims 36 and 37.

III. Rejections under 35 U.S.C. § 112, first paragraph

a. The Examiner has rejected Claims 36 and 37 under 35 U.S.C. § 112, first paragraph, alleging that they contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make the invention. More particularly, the Examiner alleges "the specification does not reasonably provide enablement for an isolated polynucleotide of claim 21 wherein said nucleotide sequence further comprises a heterologous nucleic acid sequence, claimed in Claims 36 and 37...Applicant discloses an isolated nucleic acid sequence of SEQ ID NO:1, encoding the full length hSLAP-2 polypeptide of SEQ ID NO:2 and complement thereof in the instant specification. Applicant has not taught how to make and/or use: any isolated polynucleotide of claim 21 wherein said nucleotide sequence further comprising a heterologous nucleic acid sequence, claimed in Claims 36 and 37. The structural and functional characteristics of said nucleic acid molecules are not defined in the claim. There does not appear to be sufficient guidance in the specification as filed as to how the skilled artisan would make the various nucleic acids recited in the instant claims. A person of skill in the art would not know which sequences are essential and which sequences are nonessential-
There is insufficient guidance to direct a person of skill in the art to select particular sequences or sequence lengths as essential for the function of nucleic acid sequence or SEQ ID NO:1 and polypeptide encoded by the amino acid sequence of SEQ ID NO: 2. Moreover, there is insufficient guidance as to which "isolated polynucleotide comprising a heterologous polynucleotide", recited in the claims 36 and 37 would maintain the same function of the polypeptide encoded by amino acid sequence of SEQ ID NO: 1..."

Applicants disagree with the Examiners allegation and assert that one skilled in the art would clearly know how to make and use the claimed invention since the instant specification provides the polynucleotide and polypeptide sequences of hSLAP-2. Such sequences would be all that a skilled artisan would be need in order to make and use the invention based upon the level of skill in the art for cloning polynucleotides, for example. The Examiner alleges that the addition of a heterologous sequence or a heterologous sequence encoding a heterologous polypeptide to the hSLAP-2 coding sequence is somehow expected to alter the function of hSLAP-2. Applicants do not agree with this allegation, nor do Applicants understand the rational behind the allegation since fusion proteins are routinely created in the art that retain, and are expected to retain, the function of the host polypeptide. However, in the interest of facilitating prosecution and in recognition of the fact that the

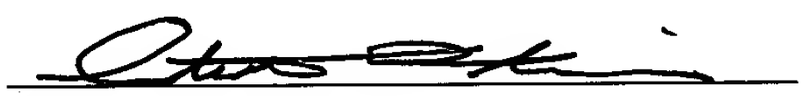
scope of Claim 21 encompasses any such heterologous sequences, in addition to any other sequences, that are appended to the sequences of Claim 21 as a consequence of the use of "comprising" language within the preamble of Claim 21, Applicants have cancelled Claims 36 and 37. Applicants believe the Examiners rejection of these Claims has been rendered moot in light of these cancellations.

Applicants believe that all of the Examiners rejections and objections have been overcome and that all of the pending claims before the Examiner are in condition for allowance. An early Office Action to that effect is, therefore, earnestly solicited.

A three-month extension is hereby requested pursuant to 37 CFR §1.136(a). Please charge Deposit Account No. 19-3880 in the name of Bristol-Myers Squibb Company in the amount of $1020 for payment of the extension fee.

If any fee is due in connection herewith not already accounted for, please charge such fee to Deposit Account No. 19-3880 of the undersigned. Furthermore, if any extension of time not already accounted for is required, such extension is hereby petitioned for, and it is requested that any fee due for said extension be charged to the above-stated Deposit Account.

Respectfully submitted,



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try and 10% of whom were of other ethnic backgrounds. The stop codon mutation was screened in 70 Finnish EPM1 carrier parents. All 70 of these individuals contained the common ancestral haplotype around the EPM1 locus on one of their chromosomes. To distinguish mutations from polymorphisms, we considered only the nonancestral haplotype chromosome of these 70 individuals. DNA from these individuals was amplified by PCR, and the products were directly sequenced with the AmpliCycle sequencing kit (Perkin-Elmer).

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Enhancement of Antitumor Immunity by CTLA-4 Blockade

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One reason for the poor immunogenicity of many tumors may be that they cannot provide signals for CD28-mediated costimulation necessary to fully activate T cells. It has recently become apparent that CTLA-4, a second counterreceptor for the B7 family of costimulatory molecules, is a negative regulator of T cell activation. Here, in vivo administration of antibodies to CTLA-4 resulted in the rejection of tumors, including preestablished tumors. Furthermore, this rejection resulted in immunity to a secondary exposure to tumor cells. These results suggest that blockade of the inhibitory effects of CTLA-4 can allow for, and potentiate, effective immune responses against tumor cells.

Despite expressing antigens recognizable by a host's immune system, tumors are very poor in initiating effective immune responses. One reason for this poor immunogenicity may be that the presentation of antigen alone is insufficient to activate T cells. In addition to T cell receptor engagement of an antigenic peptide bound to major histocompatibility complex (MHC) molecules, additional costimulatory signals are necessary for T cell activation (*1*). The most important of these costimulatory signals appears to be provided by the interaction of CD28 on T cells with its primary ligands B7-1 (CD80) and B7-2 (CD86) on the surface of specialized antigen-presenting cells (APCs) (*2–4*). Expression of B7 costimulatory molecules is limited to specialized APCs. Therefore, even though most tissue-derived tumors may present antigen in the context of MHC molecules, they may fail to elicit effective immunity because of a lack of costimulatory ability. Several studies support this notion. In a variety of model systems, transfected tumor cells expressing costimulatory B7 molecules induced potent responses against both modified and unmodified tumor cells (*5–8*). It appears that tumor cells transfected with B7 are able to behave as APCs, presumably allowing direct activation of tumor-specific T cells.

Recent evidence suggests that costimulation is more complex than originally thought and involves competing stimulatory and inhibitory signaling events (*3, 9–12*). CTLA-4, a homolog of CD28, binds both B7-1 and B7-2 with affinities much greater than does CD28 (*13–16*). In vitro, antibody cross-linking of CTLA-4 has been shown to inhibit T cell proliferation and interleukin-2 production induced by antibody to CD3 (anti-CD3), whereas blockade of CTLA-4 with soluble intact or Fab fragments of antibody enhances proliferative responses (*17, 18*). Similarly, soluble intact or Fab fragments of anti–CTLA-4 greatly augment T cell responses to nominal peptide antigen or the superantigen *Staphylococcus* enterotoxin B in vivo (*19, 20*). It has also been suggested that CTLA-4 engagement can induce apoptosis in activated T cells (*21*). Finally, mice deficient in CTLA-4 exhibit severe T cell proliferative disorders (*22*). These results demonstrate that CTLA-4 is a negative regulator of T cell responses and raise the possibility that blockade of inhibitory signals delivered by CTLA-4–B7 interactions might augment T cell responses to tumor cells and enhance antitumor immunity.

We first sought to determine whether CTLA-4 blockade with nonstimulatory, bivalent antibody (*18, 20*) would accelerate rejection of B7-positive tumor cells. Previously, we showed that B7-1 expression was partially successful at inducing rejection of the transplantable murine colon carcinoma 51BLim10 (*23*). We reasoned that CTLA-4 blockade would remove inhibitory signals in the costimulatory pathway, resulting in enhanced rejection of the tumor cells. We injected groups of BALB/c mice with B7-1–transfected 51BLim10 tumor cells (B7-51BLim10) (*23*). Two groups were treated with a series of intraperitoneal injections of either anti–CTLA-4 or anti-CD28 (*18, 24*). Treatment with anti–CTLA-4 inhibited B7-51BLim10 tumor growth as compared with the anti-CD28–treated mice or the untreated controls (Fig. 1). All mice in the untreated and anti-CD28–treated groups developed small tumors that grew progressively for 5 to 10 days and then ultimately regressed in 8 of the 10 mice by about day 23 after injection. The two small tumors that did not regress remained static for more than 90 days. In contrast, three of five mice treated with anti–CTLA-4 developed very small tumors, all of which regressed completely by day 17. Although these results were encouraging and were consistent with our hypothesis, they were not very dramatic because B7-1 expression resulted in fairly rapid rejection of transfected 51BLim10 cells even in the absence of CTLA-4 blockade; however, these results confirmed that anti–CTLA-4 did not inhibit tumor rejection.

We next examined the effects of CTLA-4 blockade on the growth of V51BLim10, a vector control tumor cell line that does not express B7 (*23*). All mice either injected with 4×10^6 V51BLim10

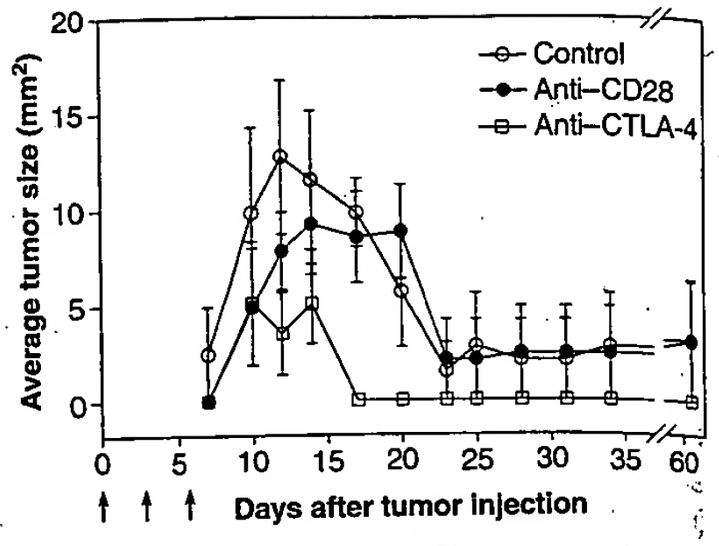

Fig. 1. Treatment with anti–CTLA-4 accelerates rejection of a B7-1–positive colon carcinoma (*23*). A volume of 100 μl of cell suspension (4×10^6 cells) was injected subcutaneously into the left flanks of groups of five female BALB/c mice. Two of the groups received three intraperitoneal injections of either anti–CTLA-4 or anti-CD28 (*18*). Injections of 100, 50, and 50 μg of antibody were given on days 0, 3, and 6, respectively, as indicated by the arrows. Control animals received no injections. Data points represent the average of the products of bisecting tumor diameters. Error bars represent standard error of the mean.

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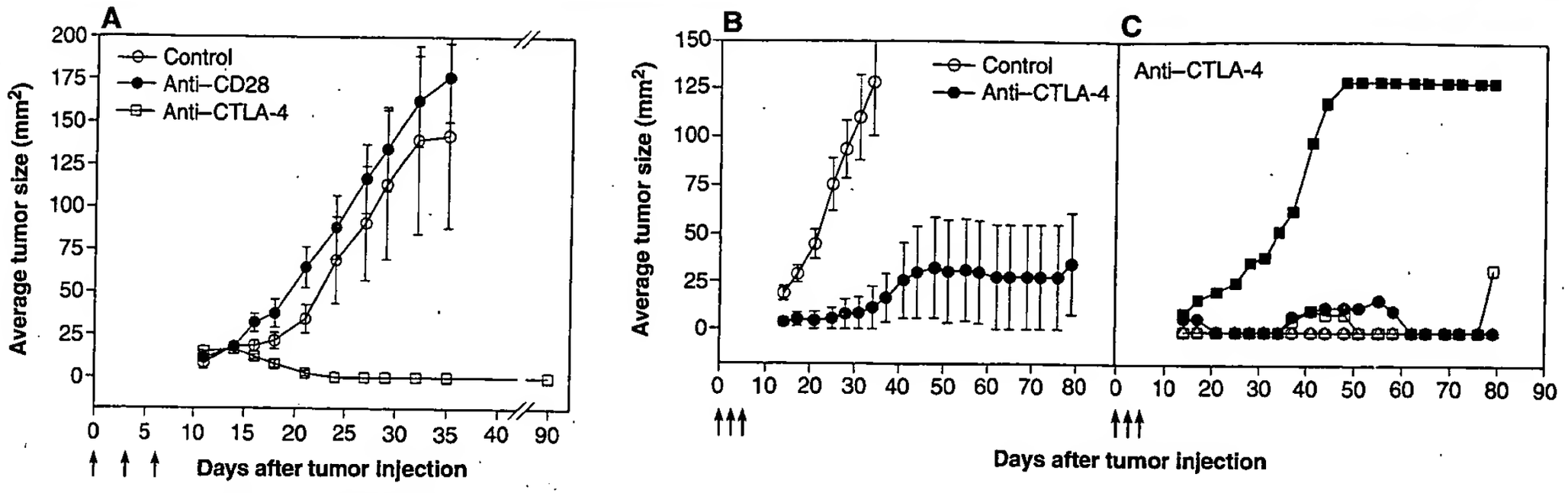

Fig. 2. Treatment with anti–CTLA-4 enhances rejection of B7-negative colon carcinoma cells and results in protection against subsequent challenge with wild-type colon carcinoma cells. Groups of BALB/c mice were injected with B7-negative 51BLim10 vector control cells (V51BLim10), left untreated, or treated with anti–CTLA-4 or control antibody. Mice were euthanized when tumors reached a size of 200 mm^2 or became ulcerated. If individual mice within a group were euthanized, the final measurement was carried over to subsequent time points. (**A**) Average tumor size in mice injected with 4×10^6 tumor cells. Groups of five mice were injected with 4×10^6 V51BLim10 tumor cells. Treated groups were injected three times with 100 μg of anti–CTLA-4 or anti-CD28 as indicated by the arrows. All untreated control and anti-CD28–treated mice were killed by day 35. Mice treated with anti–CTLA-4 remained tumor-free for more than 90 days. Error bars represent standard error of the mean. (**B**) Average tumor size in mice injected with 2×10^6 V51BLim10 tumor cells. Two groups of five mice were injected with tumor cells and treated as above with anti–CTLA-4 or irrelevant hamster antibody. (**C**) Individual tumor growth in mice injected with 2×10^6 V51BLim10 cells and treated with anti–CTLA-4. Three of the mice remained tumor-free beyond 80 days. (**D**) Challenge tumor growth in anti–CTLA-4–treated mice. Five anti–CTLA-4–treated mice that had completely rejected V51BLim10 tumor cells were rechallenged 70 days later with 4×10^6 wild-type tumor cells injected subcutaneously in the opposite flank. Five naïve mice were also injected as controls. All control mice developed progressively growing tumors and were euthanized on day 35 after inoculation. Three of five previously immunized mice remained tumor-free 70 days after rechallenge.

tumor cells and left untreated, or treated with anti-CD28, developed progressively growing tumors and required euthanasia by 35 days after inoculation (Fig. 2A). In contrast, all mice treated with anti–CTLA-4 completely rejected their tumors after a short period of limited growth. Similarly, control mice injected with 2×10^6 tumor cells developed rapidly growing tumors and required euthanasia by day 35 (Fig. 2B). Anti–CTLA-4 treatment had a dramatic effect on tumor growth, but one mouse did develop a tumor quickly (accounting for a majority of the growth indicated in Fig. 2B) and another developed a tumor much later (Fig. 2C). Anti–CTLA-4 appeared to be less effective at a tumor dose of 1×10^6 cells, where treatment resulted in significantly reduced tumor growth rates, but four of five mice developed progressively growing tumors (25). Thus, although curative responses were not obtained in all cases, it is clear that CTLA-4 blockade significantly enhanced rejection of B7-negative tumor cells.

We next sought to determine whether tumor rejection as a consequence of CTLA-4 blockade was associated with enhanced immunity to a secondary challenge. Mice that had rejected V51BLim10 tumor cells as a result of treatment with anti–CTLA-4 were challenged with 4×10^6 wild-type 51BLim10 cells 70 days after their initial tumor injections. These mice showed significant protection against a secondary challenge as compared with naïve controls (Fig. 2D). All control animals had progressively growing tumors by 14 days after injection, developed massive tumor burdens, and required euthanasia by day 35. Only one of the previously immunized mice had a detectable tumor by day 14, and growth of this tumor was very slow. Ultimately, two more tumors developed in the immunized mice 42 days after challenge. Two mice remained tumor-free throughout the course of the experiment. These results demonstrate that tumor rejection mediated by CTLA-4 blockade results in immunologic memory.

To determine whether anti–CTLA-4 treatment could have an effect on the growth of established tumors, we injected groups of mice with 2×10^6 wild-type 51BLim10 tumor cells and treated them with anti–CTLA-4 beginning on day 0 as before, or beginning 7 days later at which time most mice had palpable tumors. Mice treated with anti–CTLA-4 at either time period had significantly reduced tumor growth compared with untreated controls (Fig. 3). In fact, delaying treatment appeared to be more effective, with two of five mice remaining tumor-free beyond 30 days after inoculation.

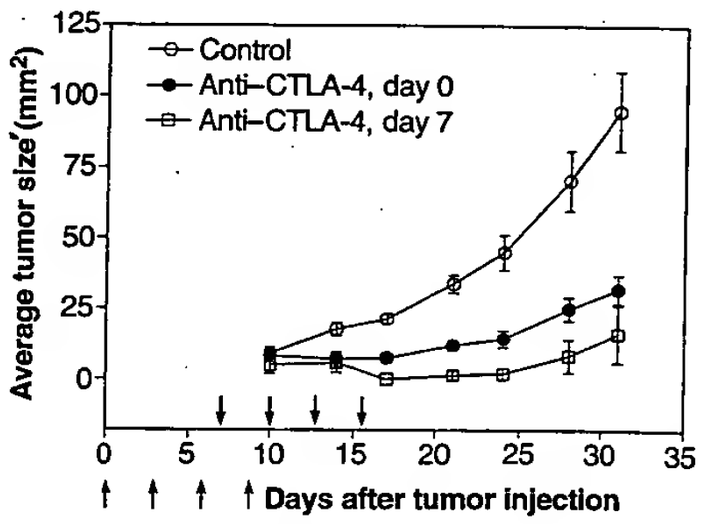

Fig. 3. Treatment with anti–CTLA-4 reduces the growth of established tumor. Groups of mice were injected subcutaneously with 2×10^6 51BLim10 tumor cells. Control animals ($n = 10$) were injected intraperitoneally with 100 μg of irrelevant hamster antibody on days 0, 3, 6, and 9, as indicated by the upward-pointing arrows. One anti–CTLA-4 treatment group ($n = 10$) received intraperitoneal injections on the same days. The other treated mice ($n = 5$) were given intraperitoneal injections of anti–CTLA-4 beginning on day 7 and subsequently on days 10, 13, and 16 (downward-pointing arrows).

The effects of anti–CTLA-4 treatment were not limited to variants of the murine colon carcinoma 51BLim10. Similar results were obtained with a rapidly growing fibrosarcoma of A/JCr mice, Sa1N (26) (Fig. 4). All control mice injected subcutaneously with 1×10^6 Sa1N cells developed measur-

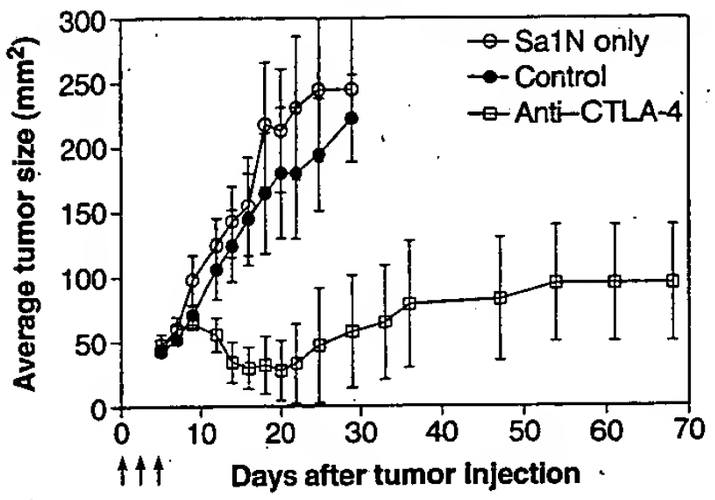

Fig. 4. Treatment with anti–CTLA-4 reduces the growth of the murine fibrosarcoma Sa1N. Groups of five mice were injected subcutaneously in the flank with a suspension of 1×10^6 Sa1N fibrosarcoma cells. Treated groups were injected intraperitoneally with 100 μg of anti–CTLA-4 or irrelevant hamster control antibody at days 0, 3, and 6 as indicated by the arrows. All control animals were killed by day 30. Two of five animals treated with anti–CTLA-4 remained tumor-free at day 55.

able, rapidly growing tumors within 7 days, whereas only two mice treated with anti–CTLA-4 had tumors by day 30, and one additional mouse developed a tumor around day 40 after injection. The remaining mice were still tumor-free 70 days after injection. In another experiment, control mice injected with 4×10^5 Sa1N tumor cells also developed rapidly growing tumors, whereas 7 of 10 mice treated with anti–CTLA-4 were tumor-free by day 25 after injection (*25*).

Our results indicate that removing inhibitory signals in the costimulatory pathway can enhance antitumor immunity. Although it has been shown that anti–CTLA-4 interferes with signals that normally down-regulate T cell responses in vivo (*17*, *18*), the exact mechanisms of antitumor immunity elicited by CTLA-4 blockade are not clear. In the case of B7-negative tumors, antigens are most likely transferred to and presented by host APCs (*27*), where CTLA-4 blockade might effect T cell responses in two nonexclusive ways. First, removal of inhibitory signals may lower the overall threshold of T cell activation and allow normally unreactive T cells to become activated. Alternatively, CTLA-4 blockade might sustain proliferation of activated T cells by removing inhibitory signals that would normally terminate the response, thus allowing for greater expansion of tumor-specific T cells.

Regardless of the mechanism, it is clear that CTLA-4 blockade enhances antitumor responses. Most importantly, we have observed these effects against unmanipulated, wild-type tumors. Current methods of enhancing antitumor immunity generally require the engineering of tumor cells (*8*). Some of these methods, such as the induction of B7 expression, rely on enhancing the costimulatory activity of the tumor cells themselves. Others, such as engineering tumor cells to express MHC class II molecules (*26*, *28*, *29*) or to produce granulocyte-macrophage colony-stimulating factor (*27*, *30*, *31*) or pulsing dendritic cells with tumor antigen ex vivo (*32*, *33*), seek to enhance antigen presentation, antigen transfer, or both. Thus, CTLA-4 blockade, by removing potentially competing inhibitory signals, may be a particularly useful adjunct to other therapeutic approaches involving the costimulatory pathway.

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Light-Induced Degradation of TIMELESS and Entrainment of the *Drosophila* Circadian Clock

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Two genes, *period* (*per*) and *timeless* (*tim*), are required for production of circadian rhythms in *Drosophila*. The proteins encoded by these genes (PER and TIM) physically interact, and the timing of their association and nuclear localization is believed to promote cycles of *per* and *tim* transcription through an autoregulatory feedback loop. Here it is shown that TIM protein may also couple this molecular pacemaker to the environment, because TIM is rapidly degraded after exposure to light. TIM accumulated rhythmically in nuclei of eyes and in pacemaker cells of the brain. The phase of these rhythms was differentially advanced or delayed by light pulses delivered at different times of day, corresponding with phase shifts induced in the behavioral rhythms.

Circadian rhythms, found in most eukaryotes and some prokaryotes (*1*), are ~24-hour rhythms governed by an internal clock that functions autonomously but can be entrained by environmental cycles of light or temperature. Circadian rhythms produced in constant darkness can also be reset by pulses of light. Such light pulses will shift the phase of the clock in different directions (advance or delay) and to a varying extent in a manner that depends on the time of light exposure (*2*).

In the fruit fly *Drosophila melanogaster*, two genes, *period* (*3*) and *timeless* (*4*), are

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Prevention and amelioration of collagen-induced arthritis by blockade of the CD28 co-stimulatory pathway: requirement for both B7-1 and B7-2

Collagen type II-induced arthritis (CIA) is an experimental model of arthritis that has been successfully used to dissect the pathogenesis of human rheumatoid arthiritis and to identify potential therapeutic targets. We have used this model to evaluate the role of T cell co-stimulation in both disease development and progression. T cell co-stimulation is provided by ligation of CD28 with either B7-1 or B7-2 present on antigen-presenting cells and can be prevented by a soluble form of CTLA-4 (CTLA-4Ig) which binds with high affinity to both B7-1 and B7-2. We found that administration of CTLA-4Ig at the time of immunization prevented the development of CIA and was associated with lack of lymphocyte expansion within the draining lymph node and failure to produce anti-collagen IgG1 or IgG2a antibodies. To determine which CD28 ligand plays a more dominant role in CIA, we treated mice with monoclonal antibodies (mAb) against either B7-1 or B7-2. Neither anti-B7-1 nor anti-B7-2 had any effect on the course of CIA when given alone, but resulted in reduced incidence and clinical scores when given together. Interestingly, when treatment was delayed until after the onset of clinical disease, both CTLA-4Ig or anti-B7-1 plus anti-B7-2 mAb still ameliorated disease. Effective treatment was associated with a reduction in interferon-γ production by lymph node cells following stimulation *in vitro*, suggesting that Th1 responses were diminished. This study points to a critical role of CD28 co-stimulation in the development and perpetuation of CIA in DBA/1 mice. Interestingly, it demonstrates an active role for T cells in the later stages of this disease and implicates both B7-1 and B7-2-mediated co-stimulation in the pathogenesis of CIA.

1 Introduction

Collagen type II-induced arthritis (CIA) is an experimental model of arthritis that can be induced in genetically susceptible rodents by immunization with native bovine type II collagen in complete Freund's adjuvant (CFA, [1–3]). The development of arthritis is associated with high levels of both cell-mediated and humoral immunity to collagen [4, 5], and is characterized by synovial hyperplasia and pannus formation with infiltration of neutrophils and mononuclear cells. In later phases of the disease, there is destruction of cartilage and bone, followed by deposition of fibrous tissue and subsequent ankylosis in the joint [6].

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Abbreviations: **CIA:** Collagen-induced arthritis **RA:** Rheumatoid arthritis **CII:** Collagen type II **EAE:** Experimental allergic encephalomyelitis **IDDM:** Insulin-dependent diabetes mellitis **DIP:** Distal interphalangeal joint **PIP:** Proximal interphalangeal joint **MTP:** Metatarsophalangeal joint

Key words: Collagen-induced arthritis / T cell / CD28 co-stimulation / CTLA-4Ig / B7-1/B7-2

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CIA has been extensively studied to elucidate the pathogenic mechanisms relevant to human rheumatoid arthritis (RA), and to identify potential therapeutic targets (reviewed in [7]). The development of CIA is known to depend on $CD4^+$ T cell activation; nude mice and rats are resistant to CIA, and disease susceptibility is linked to the MHC region and the presence of certain T cell receptor (TCR) genes [8–10]. Arthritis can be prevented by treatment around the time of collagen immunization with mAb to CD4, TCR, MHC class II or CD25 [11–15]. Following T cell activation, an inflammatory cascade involving T cells, macrophages/monocytes, synoviocytes, cytokines and growth factors is triggered which leads to the characteristic joint destruction of arthritis [16]. The role of T cells during this effector phase of disease is less well defined. Although treatment of mice with anti-CD4 mAb post-disease onset causes some reduction in paw swelling, it has little effect on the histological outcome of arthritis [13]. In human RA, anti-CD4 mAb therapy has not given significant clinical benefits. This may be due to an inability to administer adequate quantities of mAb to modulate T cell function or could reflect a minimal role for T cells in established RA [17–20].

$CD4^+$ T helper (Th) cells have been divided into subsets, termed Th1 and Th2, depending upon their cytokine profile [21]. Th1 cells are recognized by their secretion of IL-2, IFN-γ, and lymphotoxin (LT), and are associated with cellular immunity and class switching to the IgG2a isotype. Conversely, Th2 cells are associated with humoral immune responses and switching to IgG1 and IgE due to their production of IL-4 and IL-5 [21]. Both Th1 and Th2 cell activity is implicated in the development of CIA since both cell-

mediated and humoral respo are required [5] and anti-CII antibodies of all isotypes (including IgE) are produced during the course of disease [22–24]. Although Th1 cells are thought to play a dominant role in some organ-specific autoimmunities such as experimental allergic encephalomyelitis (EAE) and insulin-dependent diabetes myelitis (IDDM) [25], the role of Th1 cells in CIA is unclear [26]. IL-12, a potent inducer of Th1 subset development, can increase or decrease the severity of CIA in DBA/1 male mice depending on the dose administered [27, 28], and the effects of IFN-γ blockade have yielded conflicting results [29, 30]. This suggests that a fine balance between Th1 and Th2 subset activity is required for CIA development.

The role of CD28 co-stimulation in the development and perpetuation of Th1 and Th2 cells is unclear. B7-1 and B7-2, the ligands for CD28, have been shown preferentially to induce Th1 and Th2 subset development, respectively [31, 32]. However, recent studies have not shown a role for either ligand in specific Th1 and Th2 development [33, 34]. Co-stimulation via CD28 provides an important signal to antigen-stimulated T cells resulting in enhanced activation and cytokine production [35]. In contrast, signaling via CTLA-4, an alternative receptor for B7-1 and B7-2, down-regulates activated T cells [36, 37]. Since CTLA-4 binds to B7-1 with greater affinity than CD28 [38], a soluble form of CTLA-4 (CTLA-4Ig) has been used to inhibit T cell co-stimulation via CD28. CTLA-4Ig treatment suppresses both allogenic and xenogenic transplant rejection, inhibits antibody responses to T-dependent antigens and prevents the development of autoimmune diseases in murine models of EAE and lupus [39–43].

Recently, treatment of BB rats with CTLA-4Ig prior to collagen immunization was shown to protect rats from developing arthritis [44]. To further our understanding of the pathogenic mechanisms involved in the development and progression of CIA, we examined the effects of CTLA-4Ig, anti-B7-1 mAb, anti-B7-2 mAb, or anti-B7-1+anti-B7-2 mAb on the clinical and histological manifestations of this disease. We show the importance of CD28 co-stimulation in the development of CIA and the need for co-stimulation mediated via *both* the B7-1 and B7-2 ligands. Treatment with either anti-B7-1 or anti-B7-2 mAb alone had no effect on the course of disease. However, mice given either CTLA-4Ig or anti-B7-1+ anti-B7-2 mAb had reduced incidence and severity of disease. This was associated with a lack of lymphocyte expansion within the draining lymph nodes and reductions in the serum levels of anti-CII IgG1 and IgG2a isotypes, indicating a block in both Th1 and Th2 subset development. In addition, we demonstrate a role for T cells in established disease by amelioration of disease with either CTLA-4Ig or the combination of mAb against B7-1 + B7-2, when administered after the onset of clinical arthritis. Lymph node cells taken from CTLA-4Ig-treated mice produced significantly less IFN-γ than control treated mice, suggesting that blockade of CD28 co-stimulation after the onset of disease results in diminished Th1 responses.

2 Materials a ethods

2.1 Reagents

Collagen type II (CII) was purified from bovine hyaline cartilage as described [45]. Mouse CTLA-4Ig and control fusion protein L6 were generously provided by Drs. Jeff Ledbetter and Peter Linsley (Bristol Myers Squibb, Seattle, WA). Anti-B7-1 (1G10) and anti-B7-2 (2D10) were generously provided by Dr. Gordon Powers (Hoffmann-La-Roche, Nutley, NJ). Control mAb anti-MAC-1 (IgG2a) and anti-HRP (IgG2b) were purchased from the American Type Culture Collection (Rockville, MD).

2.2 Induction of arthritis and treatment protocol

Male DBA/1 mice (8–12 weeks old) were immunized with 200 μg bovine CII emulsified in CFA (Difco, Detroit, MI) by intradermal injection at the base of the tail.

For assessment of CTLA-4Ig and mAb treatment on the development of arthritis, mice were injected intraperitoneally with 100 μg mAb or fusion protein on the day of immunization and every other day until the experiment was terminated. Mice were examined daily and monitored for a further 10 days after the onset of clinical disease. The number of affected joints, clinical score and paw swelling were measured. For the clinical score, a scoring system was used where 0 = normal, 1 = slight swelling, 2 = pronounced edematous swelling and 3 = joint rigidity. Each limb was graded, resulting in a maximum score of 12 per animal. Paw swelling was assessed using calipers to measure the thickness of each affected hind paw.

For assessment of treatment initiated after disease onset, mice were examined daily and each mouse that exhibited erythema, paw swelling or both in one or more limbs was randomly assigned to a treatment or control group and given 100 μg mAb or fusion protein intraperitoneally on the first day of arthritis and subsequently treated every other day. Arthritis was then monitored for 10 days as described above.

2.3 Anti-collagen antibody ELISA

Measurement of anti-CII IgG1 and IgG2a subclasses was by a modification of an ELISA described previously [45]. Briefly, microtiter plates (Nunc, Uxbridge, GB) were coated with 2 μg/ml bovine CII dissolved in 0.05 M Tris-HCl pH 7.4 and 0.2 M NaCl overnight, blocked and then incubated with serially diluted test sera. Titrated, affinity-purified mouse anti-CII IgG was used as standard. Bound IgG1 or IgG2a was detected by incubation with alkaline phosphatase-conjugated sheep anti-mouse IgG1 or IgG2a (Binding Site, Birmingham, GB), followed by substrate (*p*-nitropohenol-phosphate). Plates were washed three times between each step with 0.01% Tween-20 PBS. Absorbance was measured at 405 nm.

2.4 Histopathology

Arthritic hind paws (the first affected hind paw of each mouse) were removed postmortem on the tenth day after onset of clinical symptoms, fixed in 10% buffered formalin, and then decalcified in 5.5% EDTA in buffered formalin. Paws were then embedded in paraffin, sectioned and stained with hematoxylin and eosin or saffranin O. Microscopic evaluation of arthritic paws was performed blinded. The severity of the arthritis in the distal interphalangeal joint (DIP), proximal interphalangeal joint (PIP) and the metatarsophalangeal joint (MTP) was classified as normal, mild, moderate or severe based on the following criteria: normal = no damage, mild = minimal synovitis, cartilage loss and bone erosions limited to discrete foci; moderate = synovitis and erosions present but normal joint architecture intact; and severe = extensive erosions and joint architecture disrupted.

2.5 Assessment of IFN-γ production

On day 3 of disease, mice were killed and inguinal lymph nodes were excised. Lymph node cells were teased apart to make a single-cell suspension, washed and live cells counted by trypan blue exclusion. Cells (2×10^5) were then placed in each well of a 96-well round-bottom plate in RPMI containing 10% heat-inactivated fetal calf serum (v/v), 100 U/ml penicillin, 100 μg/ml streptomycin, 2×10^{-5} M 2-mercaptoethanol, and 1% v/v glutamine. Cells were cultured alone or in the presence of either 5 μg/ml CII or 5 μg/ml anti-CD3 mAb (2C11-145) for 72 h. Supernatants were collected and analyzed by ELISA as described [46].

2.6 Statistical analysis

For statistical analysis of macroscopic data the Mann-Whitney U test of significance was applied using the Minitab computer package (Minitab Inc., PA).

3 Results

3.1 Development of CIA depends upon both B7-1- and B7-2-mediated co-stimulation

To examine the role of B7-1- and B7-2-mediated T cell co-stimulation in the development of CIA, two separate and concordant experiments were performed using 10 or 15 mice for each treatment group per experiment. Mice were given either anti-B7-1 mAb, anti-B7-2 mAb, a combination of both anti-B7-1 + anti-B7-2, or the fusion protein CTLA-4Ig. Controls consisted of mice treated with either the control mAb (IgG2a or IgG2b), PBS, or the fusion protein L6. Mice were injected on the day of immunization and every other day until either 10 days after the onset of clinical disease or until the experiment was terminated on day 42 after immunization. Mice treated with either the IgG2a or IgG2b control mAb, or the control fusion protein L6, developed a normal arthritis that did nor differ from that of mice treated with PBS. For clarity this data is not shown. In the first experiment, one of ten mice treated with CTLA-4Ig developed clinical signs of arthritis (Table 1). This arthritis was very late (day of onset = 42 compared with the control mean day of onset = 23) and mild (clinical score of 1). There was no detectable levels of anti-CII IgG in the serum from this mouse and histological examination revealed localized tissue inflammation rather than joint inflammation. It is unlikely that the paw swelling was in fact due to CIA. Anti-B7-1 or anti-B7-2 treatment had no effect on disease development, incidence, day of onset, severity or the number of affected joints (Table 1 and data not shown).

A second experiment was performed using the same treatment groups as above with an additional group where 15 mice were treated with a combination of both anti-B7-1 and anti-B7-2 mAb. Again, there was no effect of treatment with either anti-B7-1 or anti-B7-2 alone when compared to control groups. However, a combination treatment of anti-B7-1 + anti-B7-2 resulted in a statistically significant lower incidence of arthritis (40% compared to 90%; $p < 0.05$), and a milder disease in arthritic animals (mean clinical score 2.9 compared to 4.9; $p < 0.05$), but no

Table 1. Effect of mAb treatment on CIA[a)]

Treatment	Incidence	Day of onset	Clinical score of diseased animals Day 3	Day 5	Day 10
First experiment					
PBS	9 of 10	22.6 ± 2.6	2.3 ± 0.4	2.7 ± 0.4	3.4 ± 0.6
Anti-B7-1	7 of 10	26.3 ± 3.1	2.9 ± 0.6	3.1 ± 0.7	3.4 ± 0.7
Anti-B7-2	8 of 10	23.6 ± 2.2	1.9 ± 0.2	2.9 ± 0.4	3.6 ± 0.3
CTLA-4Ig	1 of 10**	42	1	1	1
Second experiment					
PBS	9 of 10	26.4 ± 1.31	3.1 ± 0.4	4.0 ± 0.5	4.9 ± 0.5
Anti-B7-1	9 of 10	24.8 ± 1.6	2.3 ± 0.4	3.9 ± 0.7	4.7 ± 0.4
Anti-B7-2	6 of 10	23.3 ± 1.4	2.0 ± 0.0	2.7 ± 0.4	4.2 ± 0.5
Anti-B7-1 + B7-2	6 of 15*	21.7 ± 1.9	2.1 ± 0.3	2.4 ± 0.2*	2.9 ± 0.4*
CTLA-4Ig	0 of 10**	NA[b)]	NA	NA	NA

a) Mice were immunized with bovine CII in CFA and injected every other day with 100 μg mAb or PBS.
* Statistically significant, $p < 0.05$.
**Statistically significant, $p < 0.001$.
b) NA: not applicable.

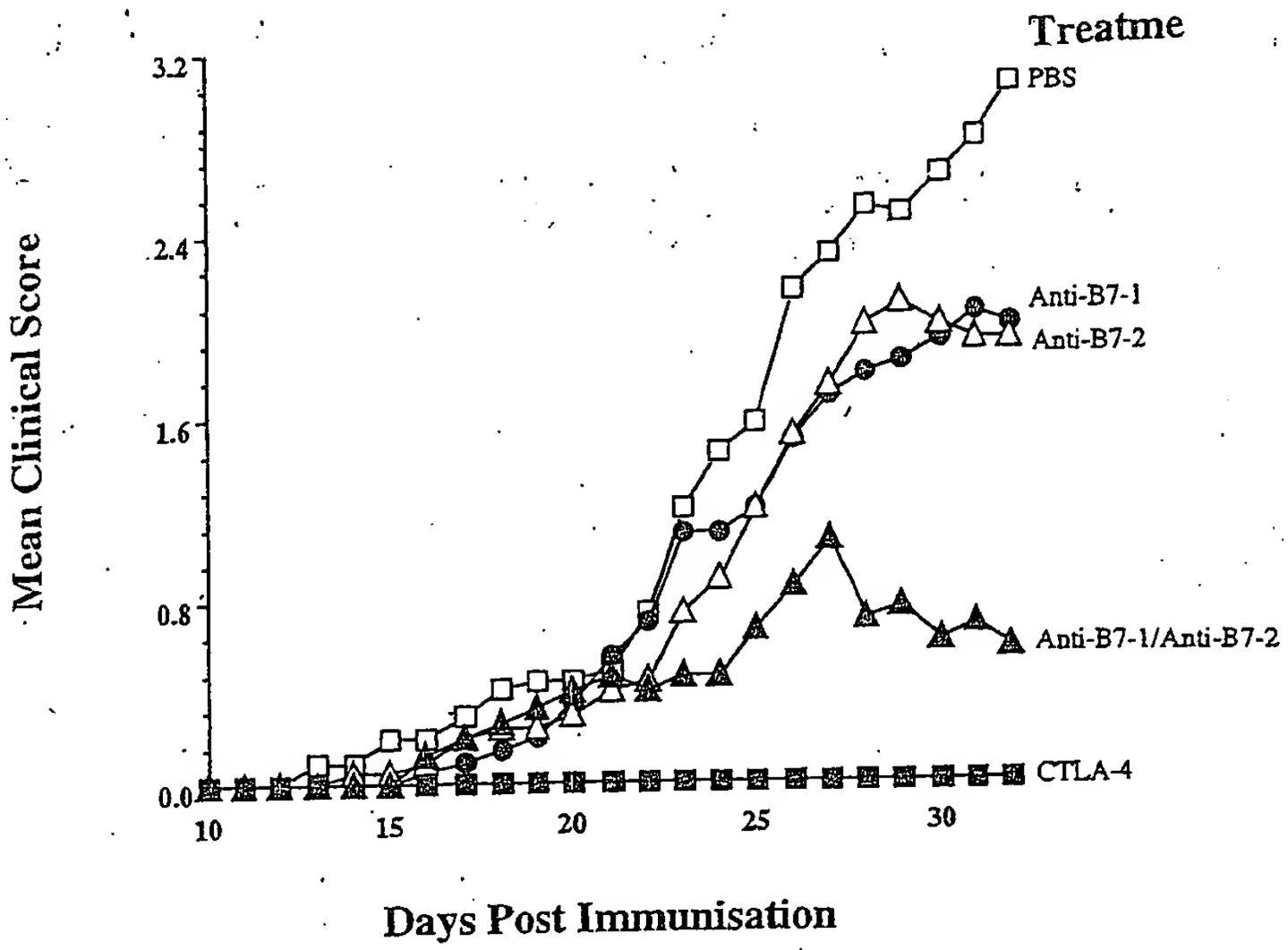

Figure 1. The Effect of mAb pretreatment on development of CIA. Mice (10 or 15) were randomly assigned to each pretreatment group and on the day of immunization were given either PBS (□), 100 μg of either anti-B7-1 (●), anti-B7-2 (△), anti-B7-1+anti-B7-2 (▲), or CTLA-4Ig (■) and every other day until day 10 of disease or day 42 when each experiment was terminated. Results are from two separate experiments and show the mean clinical scores of all mice in each pretreatment group during 32 days.

delay on the day of disease onset. CTLA-4Ig treatment prevented development of disease in all the treated mice ($p < 0.001$) for a period of up to 42 days post-immunization. The results from these two experiments were pooled and the mean clinical scores of all animals in each treatment group calculated (Fig. 1). It is clear that both B7-1- and B7-2-mediated CD28 co-stimulation is required for the development of CIA, since only mice treated with a combination of anti-B7-1 + anti-B7-2 mAb or CTLA-4Ig showed a reduction in mean clinical scores. However, the combination treatment of anti-B7-1 + anti-B7-2 was not as effective as CTLA-4Ig treatment (Fig. 1).

To assess the extent of joint destruction following pretreatment with either PBS, control mAb, fusion protein L6, anti-B7-1, anti-B7-2, anti-B7-1+anti-B7-2, or CTLA-4Ig, the first affected paw was removed postmortem on either the tenth day of disease or on day 42 post-immunization. Microscopic analysis of hematoxylin and eosin- or saffranin O-stained sections was performed blindly. The severity of joint damage in DIP, PIP, and MTP joints was classified as either normal, mild, moderate, or severe based on the criteria described in Sect. 2.4. Histology results for each experiment are tabulated (Table 2) and data pooled and graphed (Fig. 2). Histological profiles of joints from the mice treated with either IgG2a, IgG2b control mAb, or the control fusion protein L6 did not differ from PBS-treated mice, so this data has not been graphed. Results obtained for the first and second experiment showed similar profiles for each treatment group (Table 2). In agreement with clinical data, CTLA-4Ig-treated mice showed no evidence of joint destruction in contrast to PBS- or control-treated animals (Fig. 2; data not shown). Mice treated with both anti-B7-1 + anti-B7-2 also showed reductions in joint damage (Fig. 2). Surprisingly, treatment with either anti-B7-1 or anti-B7-2 alone did cause a slight reduction in joint damage when compared to PBS, control mAb, or fusion protein (Fig. 2 and data not shown).

Table 2. Joint histology of mice pretreated with mAb[a)]

Treatment	Number of joints graded (%) Normal	Mild	Moderate	Severe
First experiment				
PBS ($n = 10$)	2 (9.5)	4 (19.0)	6 (28.6)	9 (42.8)
Anti-B7-1 ($n = 10$)	7 (46.7)	5 (33.3)	2 (13.3)	1 (6.7)
Anti-B7-2 ($n = 9$)	9 (33.3)	4 (14.8)	5 (18.5)	9 (33.3)
CTLA-4Ig ($n = 10$)	25 (100.0)	0	0	0
Second experiment				
PBS ($n = 9$)	2 (5.11)	6 (15.4)	6 (15.4)	25 (64.1)
Anti-B7-1 + B7-2 ($n = 12$)	20 (62.5)	7 (21.9)	3 (9.4)	2 (6.2)
CTLA-4Ig ($n = 10$)	25 (100.0)	0	0	0

a) Histological assessment of interphalangeal joints of mice treated with CTLA-4Ig, a combination of anti-B7-1+ anti-B7-2, anti-B7-1 only, anti-B7-2 only, or PBS. The first affected paw from each mouse was removed post-mortem 10 days after the development of arthritis or at the termination of the experiment on day 42 after immunization and examined for severity of joint damage as described in Sect. 2.4.

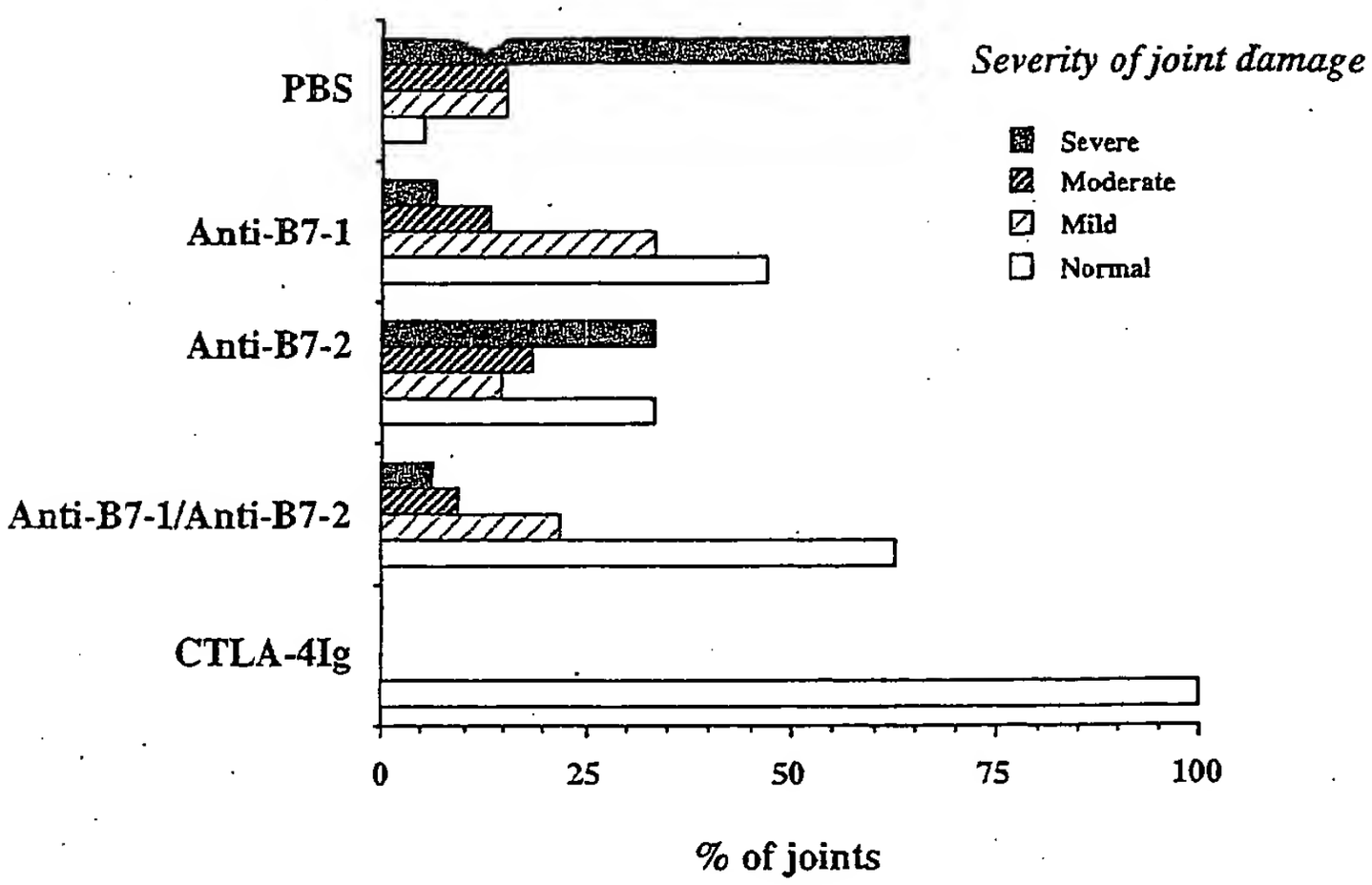

Figure 2. Histological assessment of the effect of pretreatment on CIA. Assessment of arthritis in interphalangeal joints from hindpaw sections (10 days after clinical onset or day 42 post-immunization for nonarthritic mice): *mild*, some synovitis, but significant erosions absent; *moderate*, synovitis and erosion present but normal joint architecture maintained; *severe*, synovitis, extensive erosion and normal joint architecture disrupted.

3.2 Effect of treatment on anti-CII IgG1 and IgG2a titers

To examine the effect of treatment on Th1 and Th2 subset development, we measured anti-CII IgG1 and IgG2a levels in the serum of mice 18 days after immunization (Fig. 3). Treatment with either anti-B7-1, anti-B7-2, or anti-B7-1+ anti-B7-2 had no effect on anti-CII IgG2a levels indicative of a normal Th1 response. In contrast, anti-CII IgG1 levels were reduced in mice treated with anti-B7-2 ($p < 0.05$), or anti-B7-1+ anti-B7-2 ($p < 0.01$). With the exception of CTLA-4Ig-treated mice, both arthritic and nonarthritic mice had raised anti-CII IgG2a titers, while nonarthritic mice had undetectable or much lower titers of anti-CII IgG1 antibodies than arthritic mice (data not shown).

CTLA-4Ig treatment resulted in profound suppression of anti-CII antibody production. Serum levels of IgG1 and IgG2a were very reduced or undetectable, as were levels of total IgG and IgM at day 18 post-immunization and significantly reduced in serum taken at day 42 post-immunization (Fig. 3 and data not shown).

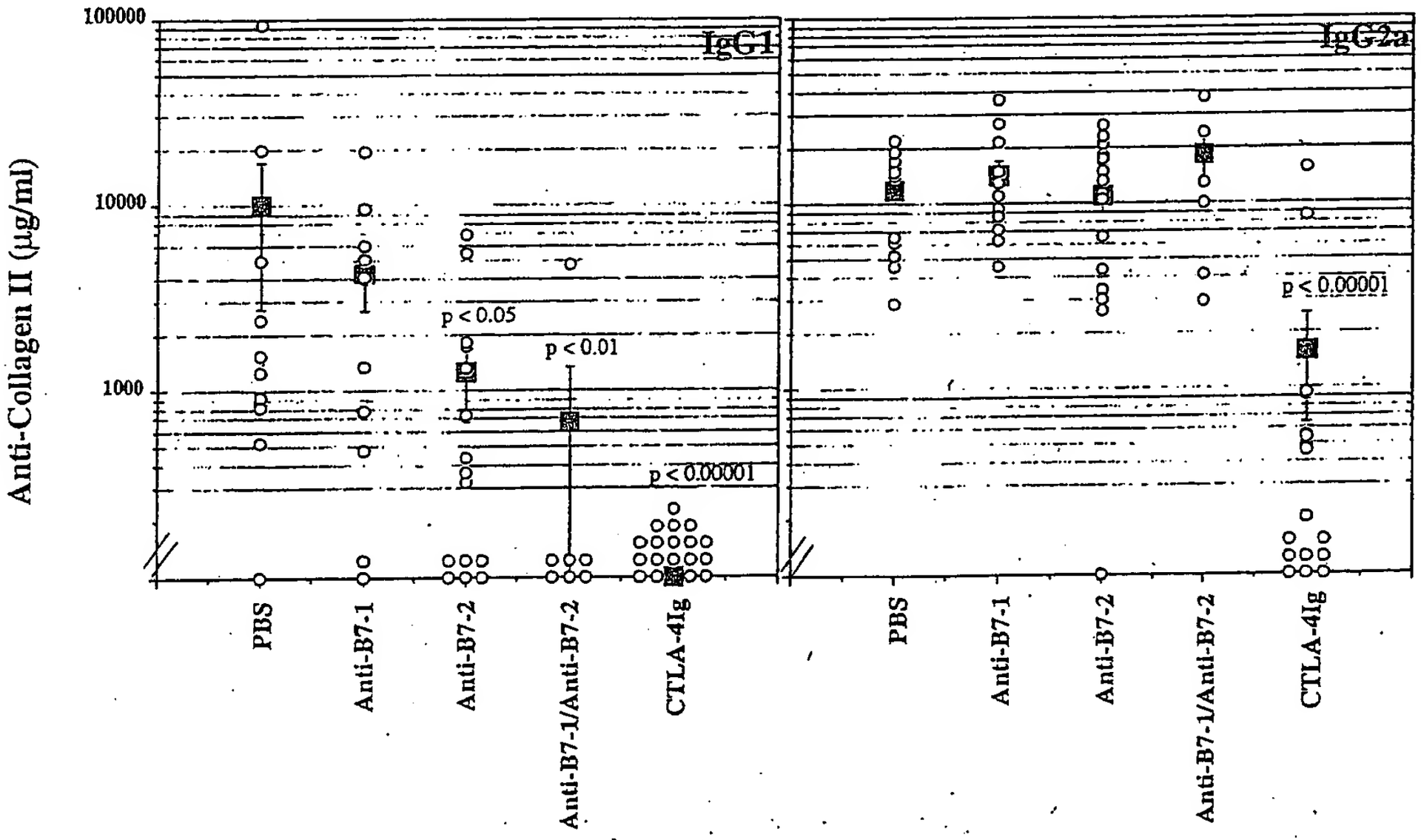

Figure 3. Effect of pretreatment on titers of anti-CII IgG1 and IgG2a antibodies. On day 18 after immunization, blood was taken from each mouse and serum levels of anti-CII IgG1 and IgG2a antibodies determined using a sandwich ELISA. Results show the individual levels of antibodies for each mouse (o) and mean levels ± SEM for each pretreatment group (▪).

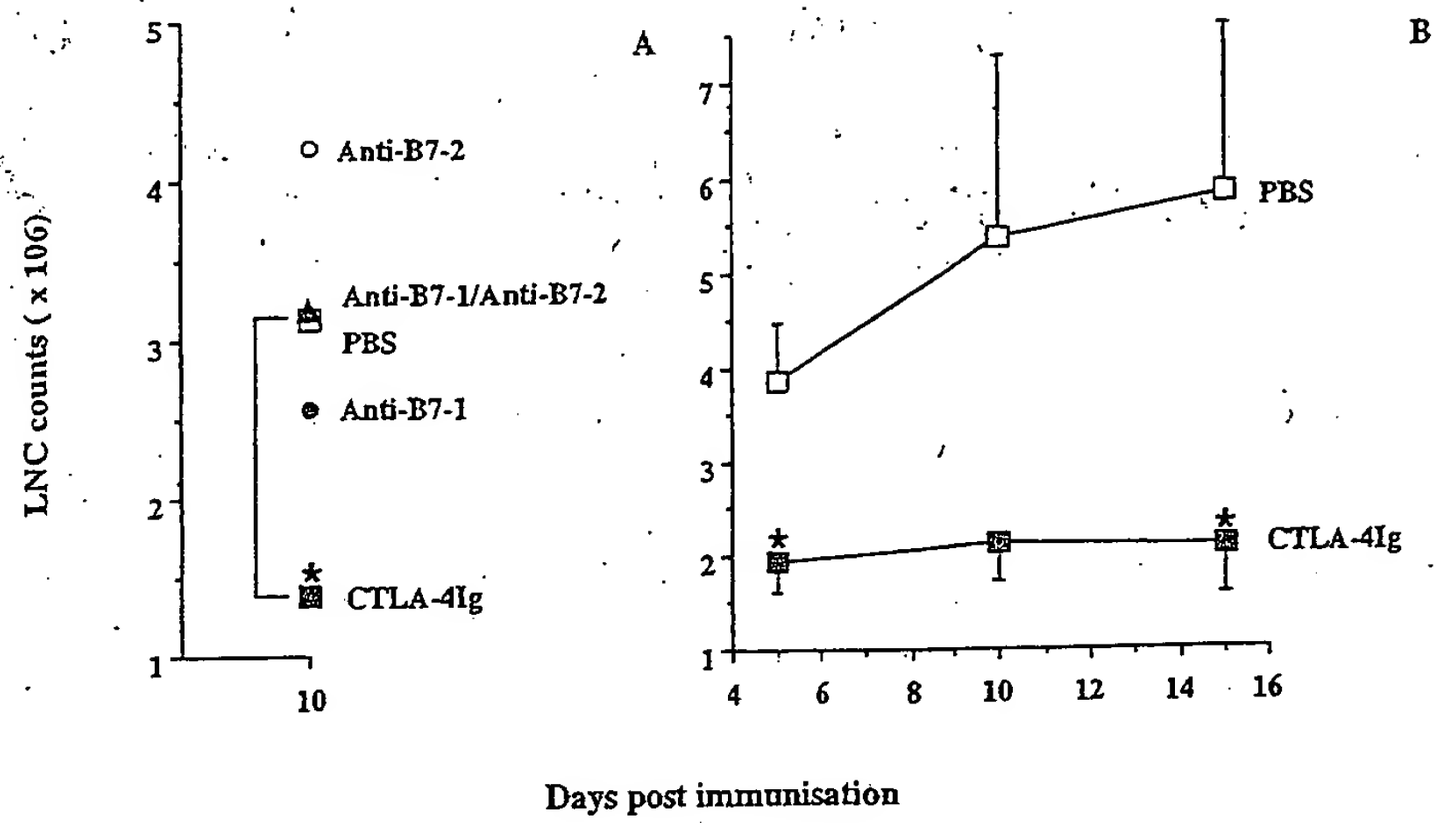

Figure 4. Effect of treatment on lymphocyte expansion. Mice were immunized and treated with PBS, anti-B7-1, anti-B7-2, anti-B7-1+ anti-B7-2, or CTLA-4Ig for a period of 10 days. The draining lymph nodes were then excised, teased apart, and counted in triplicate by trypan blue exclusion. (A) shows the mean number of lymph node cells per mouse for five mice per treatment groups ($*p < 0.05$). (B) shows the mean number of lymph node cells taken on days 5, 10, and 15 post-immunization for six mice per treatment group ± SEM ($* < 0.05$).

3.3 Blockade of CD28 co-stimulation *in vivo* inhibits expansion of lymph node cells

To assess the effect of anti-B7-1, anti-B7-2, anti-B7-1+ anti-B7-2, or CTLA-4Ig treatment prior to disease onset on lymphocyte expansion, mice were killed on day 10 after immunization and the number of live cells (determined by trypan blue exclusion) from the inguinal lymph nodes were counted in triplicate. Mice treated with either anti-B7-1 or anti-B7-2 showed no reduction in the total number of lymph node cells. However, reductions were seen in mice treated with CTLA-4Ig ($p < 0.05$, $n = 5$; Fig. 4A). We further analyzed the effect of CTLA-4Ig on lymphocyte expansion by killing mice on days 5, 10, and 15 after immunization. Treatment with CTLA-4Ig resulted in a failure of lymphocytes within the draining lymph nodes to expand over a 15-day period ($p < 0.05$, $n = 5$; Fig. 4B).

3.4 Effect of blockade of CD28 co-stimulation after disease onset

There are therapeutic modulators, such as anti-CD4 mAb, which are effective as a pretreatment, but not after the onset of CIA. Thus, it is of interest to investigate CD28 blockade after disease onset. On the first day of clinical disease, mice were randomly assigned to a treatment group and injected intraperitoneally on days 1, 3, 5, 7 and 9 of disease with either anti-B7-1, anti-B7-2, anti-B7-1+ anti-B7-2, CTLA-4Ig, control mAb, control fusions proteins, or PBS. Each joint was scored and measured and the number of affected joints noted. Pooled results from two separate experiments are depicted (Fig. 5). Mice treated with either IgG2a, IgG2b control mAb, or control fusion protein L6 did not differ in any of the parameters measured from PBS-treated mice (data not shown). Mice treated with either anti-B7-1 or anti-B7-2 alone showed no reduction in either clinical score, paw swelling, or the number of affected joints. In contrast, mice treated with either a combination of anti-B7-1 + anti-B7-2 or CTLA-4Ig showed statistically significant reductions in the clinical score, paw thickness, and the number of affected joints from day 5 onwards (Fig. 5).

The reduction in clinical scores for CTLA-4Ig and anti-B7-1+ anti-B7-2-treated animals was confirmed by histological analysis of the first affected joint which was removed postmortem on the tenth day of disease. Histology results are shown in Table 3. The histology of joints taken from mice treated with either IgG2a, IgG2b control mAb, or the control fusion protein L6 did not differ from PBS-treated mice and so have not been graphed. Histology results for mice treated with either anti-B7-1 or anti-B7-2 alone is not shown since these mice showed no evidence of clinical benefit. Mice treated with either CTLA-4Ig or the combination of anti-B7-1+anti-B7-2 showed much less joint damage than PBS, control mAb, or fusion protein-treated animals (Table 3; data not shown), although CTLA-4Ig treated mice appeared to have less joint destruction than those treated with anti-B7-1+anti-B7-2.

3.5 Blockade of CD28 co-stimulation post-disease onset reduces IFN-γ production

On day 3 of clinical disease, mice were killed and inguinal lymph node cells excised. Lymph node cells taken from mice treated with CTLA-4Ig produced significantly less IFN-γ in response to CII or anti-CD3 as compared to production by lymph node cells taken from control treated mice ($p < 0.03$; Fig. 6). In contrast, production of the Th2 cytokine IL-5 was unaltered by treatment. This suggests that effective treatment of CIA is associated with a diminished Th1 response.

4 Discussion

To determine the role of CD28 co-stimulation in the CIA model of RA, we repeatedly treated DBA/1 mice with a variety of mAb against components of the CD28 co-stimulatory pathway. Mice were treated with either anti-B7-1, anti-B7-2, a combination of anti-B7-1+anti-B7-2, CTLA-4Ig, or control mAb and fusion proteins. Our first experiments examined the role of CD28 co-stimulation in the development of disease by treating mice on the day of immunization and every other day until 10 days after disease onset or day 42 post-immunization for the nonarthritic mice. Complete blockade of CD28 co-stimulation by CTLA-4Ig abrogated development of arthritis in 19 of 20 mice. The one mouse that showed clinical evidence of very mild arthritis was subsequently found to lack synovitis or

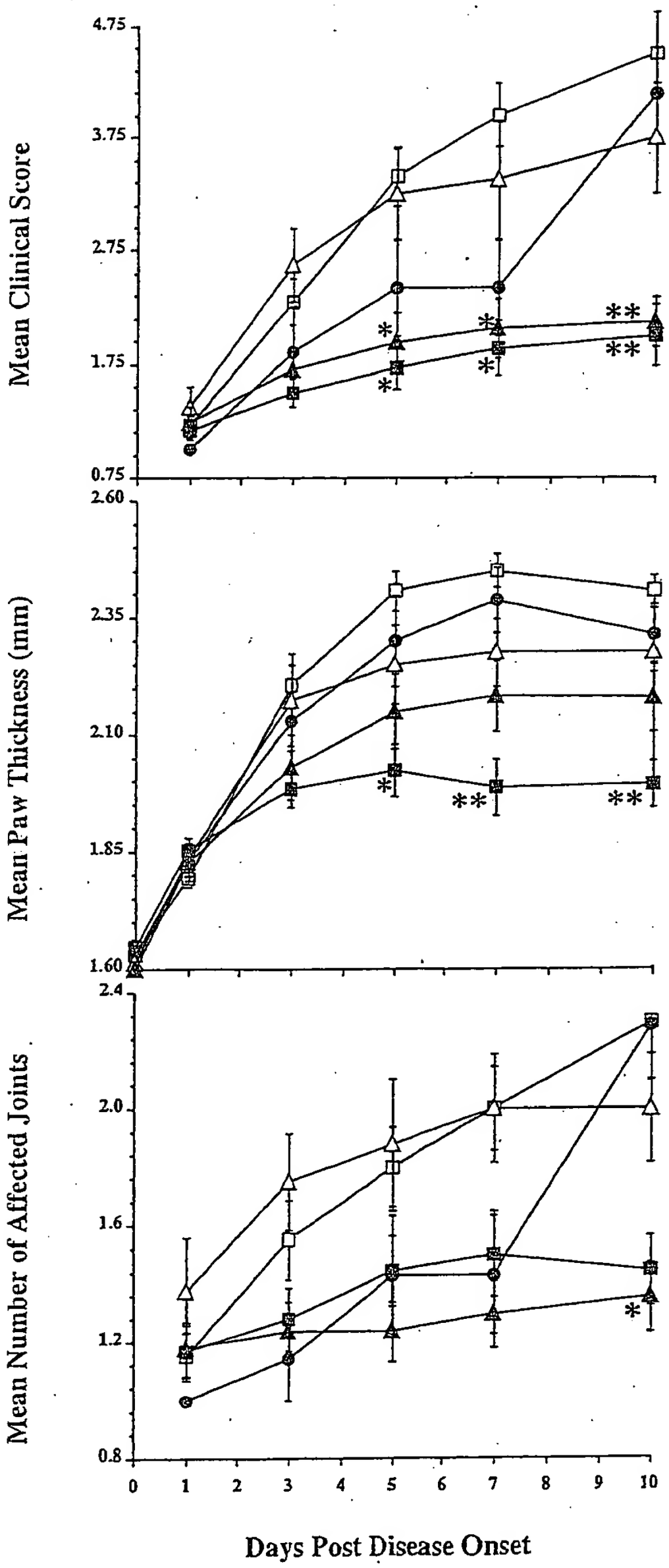

Figure 5. Effect of treatment on the progression of CIA. Mice were immunized and monitored every day for arthritis development. On the first day of clinical disease, mice were randomly assigned to a treatment group and given either PBS (□), or 100 μg of either anti-B7-1 (●), anti-B7-2 (△), anti-B7-1+anti-B7-2 (▲), or CTLA-4Ig (■) and again every other day until day 10. Results show: (upper panel) mean clinical scores ± SEM for each treatment group, (middle panel) mean paw thickness ± SEM for each treatment group, and (lower panel) mean number of affected joints ± SEM for each treatment group. $^*p < 0.0001$ (*vs.* PBS treated group), $^{**}p < 0.00001$ (*vs.* PBS treated group). CTLA-4Ig, n=18; PBS, n=20; anti-B7-1, n=7; anti-B7-2, n=8; anti-B7-1 + anti-B7-2, n=17.

Table 3. Joint histology following mAb. treatment after disease onset[a)]

Treatment	Number of joints graded (%) Mild	Moderate	Severe
First experiment			
PBS (n = 7)	2 (12.5)	2 (12.5)	12 (75)
Anti-B7-1 + anti-B7-2 (n = 3)	2 (25)	2 (25)	4 (50)
CTLA-4Ig (n = 6)	7 (58.3)	2 (16.7)	3 (25)
Second experiment			
PBS (n = 3)	1 (12.5)	2 (25)	5 (62.5)
Anti-B7-1 + anti-B7-2 (n = 5)	4 (30.8)	5 (38.5)	4 (30.8)
CTLA-4Ig (n = 4)	5 (62.5)	0 (0)	3 (37.5)

a) Histological assessment of interphalangeal joints of mice treated with CTLA-4Ig, a combination of anti-B7-1+anti-B7-2, or PBS. The first affected paw from each mouse was removed postmortem 10 days after the development of arthritis and examined for severity as described in Sect. 2.4.

joint erosion and had undetectable levels of serum anti-CII IgG. It is questionable whether this mouse had true CIA. Neither anti-B7-1 nor anti-B7-2 treatment affected CIA development unless given together. This suggests that both B7-1 and B7-2-mediated co-stimulation is required for CIA development. This differs from the results obtained in EAE and IDDM, where treatment with anti-B7-1 inhibited EAE development and treatment with anti-B7-2 inhibited IDDM development [31, 47]. In EAE, it was shown that treatment with anti-B7-2 resulted in reduced Th2 subset development and exacerbated disease, while anti-B7-1 treatment reduced the incidence of disease and resulted in the generation of predominantly Th2 cell

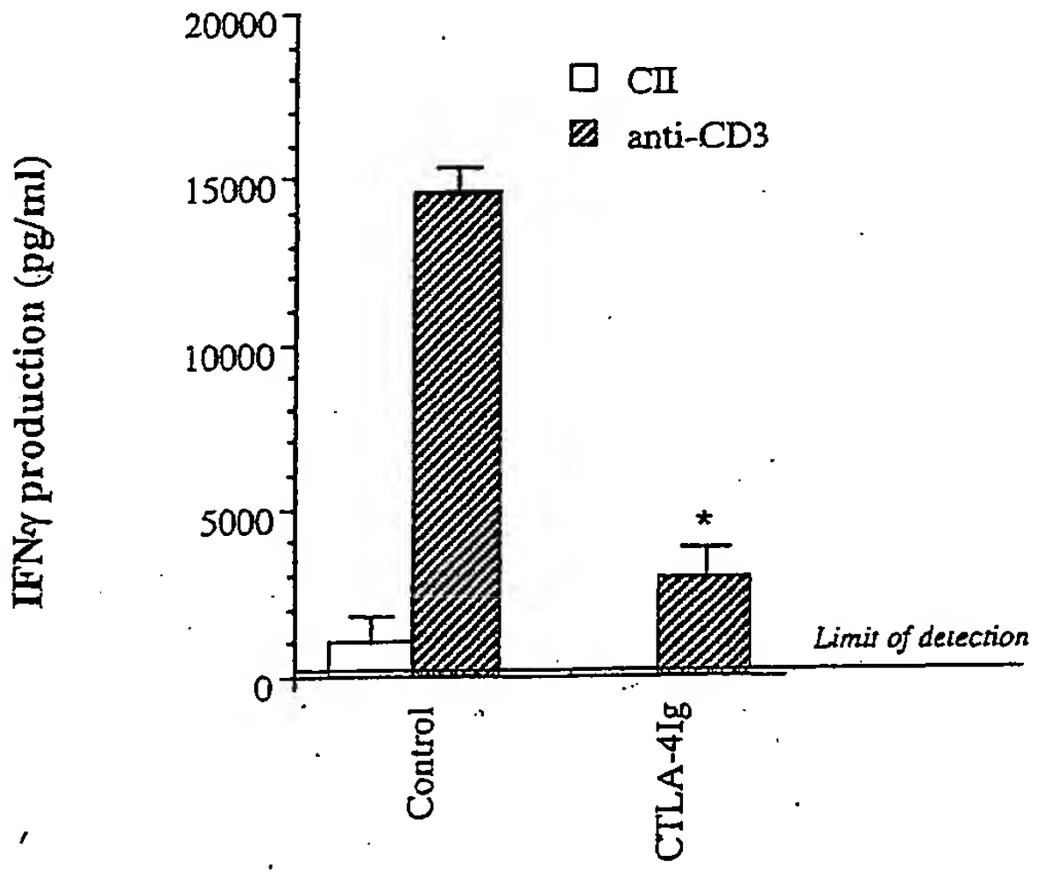

Figure 6. Effect of post-onset treatment on IFN-γ production by draining lymph node cells. On day 3 of disease, mice were killed and inguinal lymph nodes excised, washed and the cells restimulated with CII or anti-CD3 for 72 h. IFN-γ production was measured by ELISA. The results show the mean levels of IFN-γ production for five mice per treatment group ± SEM. $^*p < 0.03$.

clones. In contrast, a recen dy has described a role for *both* B7-1 and B7-2 co-stimu. ion in development of EAE [48]. The differences between these studies are currently unresolved. They could reflect different roles for B7-1 and B7-2 in different autoimmune disease models, or their different expression patterns between different strains of mice (*e.g.* SJL/J mice have higher endogenous levels of B7-1 on splenic APC than B6 mice [49]).

Since a role for B7-2 in Th2 subset development has been suggested, we examined the effect of treatment on Th subset development by measuring serum titers of anti-CII IgG1 (indicative of Th2 subset activity) and IgG2a (indicative of Th1 subset activity) 18 days after immunization. The only change in titers of anti-CII IgG2a antibodies were seen in mice treated with CTLA-4Ig. These mice had marked reductions in total anti-CII IgG and IgM antibodies (data not shown) indicating a complete block in T cell responses and not a specific block in Th1 subset development. Reductions in IgG1 titers were seen in mice treated with anti-B7-2 ($p < 0.05$), anti-B7-2+anti-B7-1 ($p < 0.001$), and CTLA-4Ig ($p < 0.00001$). The more efficacious treatments caused greater reductions Th2 subset activity since mice that did not develop arthritis had undetectable or marked reductions in IgG1 titers compared to arthritic mice (data not shown). The reductions seen in anti-CII IgG1 titers compared to arthritic mice where CD28 co-stimulation was blocked demonstrates a block in Th2 responses. We and others have shown that CD28 co-stimulation is required for Th2 subset development [50, 51] so this result was not surprising. Our data did not provide evidence that co-stimulation by either B7-1 or B7-2 alone play selective roles in the development of either Th1 or Th2 subsets. Although anti-B7-2 mAb treatment resulted in reduced IgG1 titers, there was no concomitant increase in IgG2a, and anti-B7-1 mAb had no effect on either anti-CII IgG1 or IgG2a titers. Our results suggest that both Th1 and Th2 subsets are involved in the pathogenesis of CIA, reflecting the roles of both cell-mediated and humoral immunity. As a greater threshold of antigen stimulation is required to initiate Th2 subset development [52, 53], the block seen in CIA development and IgG1 class switching probably reflects suboptimal activation of T cells, rendering them unable to induce CIA development.

Interestingly, effective pre-onset treatment correlated with a reduction in lymphocyte expansion, with CTLA-4Ig treatment having the greatest impact. This is in agreement with work showing that antigen-driven proliferation and phenotype conversion of naive $CD4^+$ T cells is dependent on CD28-derived signals *in vivo* [54]. However, recent work in a model of autoimmune oophoritis showed that neither CTLA-4Ig or anti-CD40L mAb administered alone completely prevented disease development or prevented clonal expansion of self-reactive T cells unless they were administered together [55]. The differences between these results may reflect the different models of autoimmunity used and the differences in protocols for CTLA-4Ig administration.

Our data suggest that in CIA, both B7-1- and B7-2-mediated co-stimulation is required for disease development, since neither anti-B7-1 mAb nor anti-B7-2 mAb prevented CIA development when administered alone. Administration of both anti-B7-1+anti-B7-2 mAb significantly reduce cidence, clinical scores, and anti-CII IgG1 productio.. However, these reductions were not as great as those seen in mice treated with CTLA-4Ig. This is most likely due to a lower efficacy of these mAb for inhibiting CD28 ligands. Unlike CTLA-4Ig, these antibodies may not completely prevent binding of B7-1 and B7-2 to CD28.

The role of T cells in the development of CIA is established, but their role in disease progression is less clear. This is partly because anti-CD4 mAb treatment has had little effect when administered *after* disease onset unless given with anti-TNF mAb [13]. Our study demonstrates that T cells play an active role in established disease as they are susceptible to inactivation by blockade of CD28 co-stimulation. CTLA-4Ig, or anti-B7-1+anti-B7-2 administered on the first day of clinical disease caused amelioration, but neither anti-B7-1 alone nor anti-B7-2 alone had any effect on established disease. This shows a role for both B7-1 and B7-2 in established disease in contrast to studies performed in other models of autoimmune diseases [31, 47]. A recent study showed that anti-B7-1 Fab fragments blocked clinical relapses and epitope spreading in EAE indicating that B7-1-mediated co-stimulation is important for persistent immune responses [49]. In our study, whole mAb was used and it is possible that signaling may have prevented possible amelioration by anti-B7-1 alone. However, this is unlikely, as a synergistic effect of combined anti-B7-1 mAb+anti-B7-2 mAb treatment was seen both before and after disease onset.

In several models of infectious disease, CTLA-4Ig administered after disease onset results in a down-regulation of Th1 cytokine production [50]. CTLA-4Ig treatment after the onset of EAE also causes amelioration of disease and a down-regulation of Th1 cytokine production within the CNS [56]. The amelioration of CIA following blockade of CD28 co-stimulation may be due to a down-regulation of Th1 cytokine production within the joint which would otherwise contribute to inflammatory mechanisms. We found that mice treated with CTLA-4Ig had reductions in IFN-γ, but not IL-5 production by inguinal lymph node cells following stimulation with either CII or anti-CD3. This suggests that Th1 responses are diminished following the blockade of CD28 co-stimulation.

This study has shown that CD28 co-stimulation mediated via both B7-1 and B7-2 is required for development of CIA. This augments our understanding of the pathogenesis of this autoimmune disease. More interestingly, we have shown that this co-stimulation pathway is important in *established* arthritis, again requiring both B7-1- and B7-2-mediated co-stimulation. This offers the possibility to develop novel strategies for the treatment of RA where synergies with other therapies directed against components of the inflammatory cascade are anticipated (reviewed in [57]).

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15. For further details, see M. Linial, N. Gunderson, M. Groudine [*Science* 230, 1126 (1985)]. Briefly, 10^8 nuclei in 210 μl of nuclei freezing buffer [25% glycerol, 5 mM magnesium acetate, 5 mM dithiothreitol, 50 mM tris-HCl (pH 8.0), 0.1 mM EDTA] were incubated for 30 min at 30°C in the presence of [α-^{32}P]UTP. Ribonuclease-free DNase was added and labeled RNA was eluted through a G-50 spin column (Boehringer-Mannheim), and 10^7 cpm were hybridized to immobilized, denatured plasmid DNA (5 μg per slot) in a volume of 0.5 ml for 36 hours at 65°C. Filters were washed twice for 30 min at 65°C in 0.1× standard saline citrate and 0.1% SDS, and then incubated for 30 min with ribonuclease A (10 μg/ml) in 2× standard saline citrate at 37°C.
16. Nuclei were prepared by dounce homogenization of transgenic pre-B cells in nuclei isolation buffer [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM tris-HCl (pH 7.4), 1 mM dithiothreitol] containing 0.15% NP-40, incubated with DNase I, and processed (39).
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33. The functionally rearranged genomic μ gene, μwt, has been described previously (*11*). For construction of ΔMAR, the 220-bp Hinf I fragment was modified by the addition of Xba I linkers and was inserted into Xba I–digested p1-27 (*35*) to create ΔMARμ-1. This substitution results in the removal of 344 bp and 426 bp from the 5′ end (X_1-H) and 3′ end (H-X_2) of the 1-kb, Eμ-containing X_{1-2} fragment, respectively. ΔMARμ-1 DNA was digested with Nde I (at −1) and Hpa I (at +2586), and the internal fragment was inserted into Nde I– and Hpa I–digested μwt to produce ΔMAR. For construction of μΔ4, the Hinf I sites flanking the 5′ and 3′ ends of the 220-bp enhancer core were converted to Not I and Eag I sites, respectively, to produce pμNE. After digestion of pμNE with Eag I, self-ligation of the large fragment yields μΔ4. For microinjection, plasmids were digested with Sal I and Xho I, and inserts were either excised from low melt agarose as described (*11*) or electroeluted in an Elutrap chamber (S & S); concentrated by butanol extraction, extracted with phenol, precipitated, and resuspended in 10 mM tris-HCl (pH 7.4) and 0.1 mM EDTA.
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35. For the detection of enhancer-proximal, Iμ transcripts, a single-stranded DNA probe 5′ end-labeled with ^{32}P was prepared by extending an oligonucleotide primer (BF-16, 5′-CAGAAGCCACAACCATACATTCCCA) that is complementary to a sequence 220 bp 3′ to X_2 with a Pst I–digested p1-27 template (containing a μwt fragment that extends from −18 to +2603): A linear polymerase chain reaction was performed by mixing 2.5 μl of heat-inactivated kinase reaction mixture, which contained 40 ng of the BF-16 oligonucleotide, with 2 μg of digested p1-27, 100 μM each of nucleoside triphosphates (dATP, dTTP, dCTP, and dGTP), 5 units of Taq DNA polymerase (Promega), and 2 μl of 10× Taq buffer (supplied by manufacturer; containing 15 mM $MgCl_2$) in a final volume of 20 μl, and subjecting the mixture to 20 rounds of thermal cycling for 1 min each at 94°C, 55°C, and 72°C. Samples were applied to a 5% polyacrylamide gel containing 50% (w/v) urea. The appropriate gel slice was excised and the probe was purified by electroelution (Elutrap). mb-1 transcripts were detected with a single-stranded antisense DNA probe, which was synthesized in a linear polymerase chain reaction from Asp718-digested $pMB1_{T3}$ (which contains the mb-1 cDNA beginning with the AUG codon at +27) and a ^{32}P-labeled oligonucleotide primer, mb1-1 (5′-CACCGTCAGGGATGGTGGACC, extending from +127 to +147) [A. Travis, J. Hagman, R. Grosschedl, *Mol. Cell. Biol.* 11, 5756 (1991)]. S1 reactions were performed as described (*11*).
36. Results shown in Fig. 1B, together with additional lanes containing 30 μg of the same RNA samples processed in parallel, were analyzed with a PhosphorImager (Molecular Dynamics). Relative levels of expression were determined by taking raw values corresponding to the amount of correctly initiated, transgene-specific mRNA, subtracting background, and dividing by the total level of expression in μwt line 4-1-4.
37. M12 B cells (2×10^7) [K. J. Kim *et al.*, *J. Immunol.* 122, 549 (1979)] were subjected to electroporation (Bio-Rad Gene Pulser; 0.25 kV, 960 μF in 0.4-cm cuvettes) at room temperature in phosphate-buffered saline containing both 20 μg of plasmid DNA linearized at a unique Xho I site at the 3′ end of the gene and 1 μg of Eco RI–linearized SV2neo. After 24 hours, cells were diluted to a density of 10^5/ml with complete RPMI medium containing Geneticin (2 mg/ml) (Gibco), and 1-ml aliquots were seeded into 24-well plates. Clones were picked 10 to 14 days later and maintained in nonselective medium.
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Treatment of Murine Lupus with CTLA4Ig

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The interaction of B7-related molecules on antigen-presenting cells with CD28 or CTLA-4 antigens on T cells provides a second signal for T cell activation. Selective inhibition of the B7-CD28 or B7–CTLA-4 interactions produces antigen-specific T cell unresponsiveness in vitro and suppresses immune function in vivo. To determine whether selective inhibition of the B7-CD28 or B7–CTLA-4 interactions could suppress spontaneous autoimmune disease, a B7-binding protein was generated by genetic fusion of the extracellular domain of murine CTLA-4 to the Fc portion of a mouse immunoglobulin G2a monoclonal antibody (muCTLA4Ig). In lupus-prone NZB/NZW filial generation (F_1) mice, treatment with muCTLA4Ig blocked autoantibody production and prolonged life, even when treatment was delayed until the most advanced stage of clinical illness. These findings suggest a possible role for human CTLA4Ig in the treatment of autoimmune diseases in humans.

Systemic lupus erythematosus (SLE) is a life-threatening autoimmune disease that is characterized by the production of diverse autoantibodies (*1*). Some of these autoantibodies cause damage directly as a consequence of their specificity (for example, autoimmune hemolytic anemia is caused by antibodies to red blood cells), whereas other autoantibodies cause damage indirectly as a consequence of the formation and deposition of immune complexes (for example, immune complex glomerulonephritis is caused by antibodies to nuclear antigens). Other studies have shown that, both in humans with SLE and in murine models for SLE, the production of pathologic autoantibodies by B cells is dependent on stimulatory influences from $CD4^+$ T cells (*1*, *2*).

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NZB/NZW F_1 (B/W) mice spontaneously develop an autoimmune disease that ely resembles SLE in humans (*1*, *2*). In past, in an effort to suppress T cell help autoantibody production, we treated lu--prone B/W mice with monoclonal an-odies (mAbs) to CD4 (*2*). Although this tegy is effective in murine lupus, trials in ividuals with rheumatoid arthritis indi-ed that even brief therapy with mAbs to 4 may cause long-lasting depletion of 4^+ T cells in humans (*3*). An alterna-strategy was suggested by studies that icated T cell activation requires at least signals (*4*–*9*). The first signal is provid-by the interaction between the T cell eptor and antigenic peptides in the con-t of class II major histocompatibility nplex antigens (MHC II) (*10*). This sig-is augmented by the interaction be-en CD4 and MHC II, and it can be cked by mAb to CD4 (*11*). The second nal is provided by other receptor-ligand rs on T cells and antigen-presenting cells PCs). In particular, the interactions be-en CD28 on T cells and B7 on activated ells and other APCs are capable of pro-ing this second signal (5–9). We under-k this study to determine whether selec-e inhibition of this interaction could sup-ss murine lupus in B/W mice.

Studies have shown that the CD28-B7 eraction can be inhibited by mAb to her CD28 or B7 (*12*). Under appropriate vitro conditions, this approach can in-ce antigen-specific T cell anergy (*9*, *12*). tempts to extend this observation to in o systems have focused on a somewhat ferent strategy that takes advantage of homology between CD28 and CTLA-4 , *14*). Like CD28, CTLA-4 is a member the immunoglobulin (Ig) superfamily). It is expressed on activated T cells, d it binds B7 with higher avidity than es CD28 (*14*). A protein encoded by netic fusion of the extracellular domain human CTLA-4 to an immunoglobulin 1 chain (CTLA4Ig) binds to B7 (*12*) d to a molecule similar to B7 (*15*–*17*). LA4Ig has been used successfully in vivo prolong acceptance of tissue allografts, to ibit B cell differentiation into Ig-secret-cells, and to suppress antibody responses T-dependent antigens (*18*–*20*). Despite success of CTLA4Ig in blocking prima-T-dependent immune responses, studies ve suggested that it would not prevent ondary immune responses (*20*). When atment was delayed for as little as 3 days er immunization, immune responses were t blocked (*20*).

We attempted to suppress murine lupus th injections three times per week of the man CTLA4Ig fusion protein. In these periments, mice had an immune response the fusion protein, and there was no beneficial effect on autoimmunity. Therefore, to circumvent the problem of host immunity to therapy, we produced a murine CTLA4Ig fusion protein composed of the extracellular domain of murine CTLA-4 linked to a murine Ig Cγ2a chain (muCTLA4Ig). The purified recombinant protein bound murine B7 with high avidity, blocked binding of CD28 to B7, and inhibited T cell activation in vitro (*21*).

We first treated groups of 15 5-month-old female B/W mice with either muCTLA4Ig [50 μg, intraperitoneally (IP) three times per week for 4 months] or L6, a control mouse IgG2a mAb against a human carcinoma antigen (*22*). Concentrations of muCTLA4Ig in sera were determined by a $B7^+$ CHO cell binding assay (*7*). This assay established that the dosing regimen maintained serum concentrations of ≥10 μg/ml. Treatment with muCTLA4Ig retarded the progression of murine lupus. Mice treated with muCTLA4Ig did not make antibodies to double-stranded DNA (dsDNA) at any time during the experiment, including the 3 months of observation after treatment was stopped (Fig. 1A). In contrast, control mice produced antibodies to dsDNA beginning at age 6 months. The geometric mean titers of antibodies to dsDNA in the two groups differed significantly from age 6 months to age 10 months ($P < 0.01$, by *t* test). After age 10 months, there were too few mice remaining in the control group for statistical comparison (Fig. 1).

Suppression of autoantibody production was accompanied by a significant reduction in the severity of lupus nephritis. When therapy was completed at age 9 months, the frequency of significant renal disease, defined as proteinuria ≥100 mg/dl, was 13% in mice treated with muCTLA4Ig, as compared with 87% in the control mice ($P < 0.01$, by χ^2 test). This improvement coincided with a significant improvement in survival (Fig. 1B). At 9 months of age, 93% of mice treated with muCTLA4Ig were still alive, as compared with only 40% of mice treated with L6 ($P < 0.01$, by χ^2 test). The benefit of muCTLA4Ig use persisted even after treatment was discontinued at age 9 months. During the ensuing 3 months after cessation of therapy, none of the mice that had received muCTLA4Ig died, whereas all but one of the mice in the control group died. Serum concentrations of muCTLA4Ig declined to <0.2 μg/ml within 2 months after treatment. Thus, the beneficial effects of muCTLA4Ig persisted despite declining serum concentrations.

To establish that our findings represented

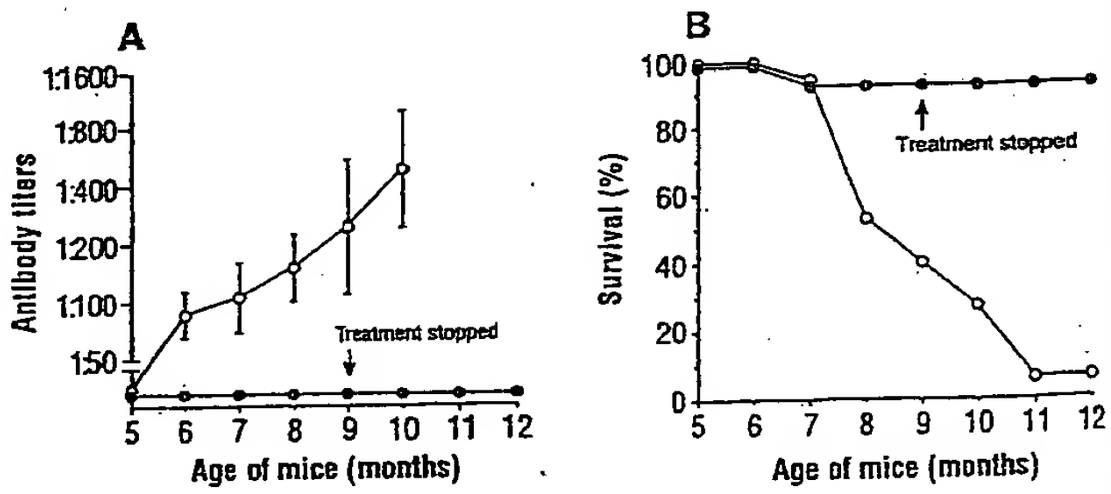

Fig. 1. (**A**) Effect of treatment with muCTLA4Ig on the titer (geometric mean ± SEM) of antibodies to dsDNA. Two groups of 15 female B/W mice were housed at the San Francisco VA Medical Center. The mice were treated with either muCTLA4Ig (closed circles) or L6 (open circles) from age 5 months to age 9 months (50 μg, IP three times per week). We analyzed sera from individual mice monthly during treatment and for 3 months thereafter, using an enzyme-linked immunosorbent assay (ELISA) as described (*2*). In this assay, the titer of antibodies to dsDNA in normal mouse serum was <1:50. (**B**) Effect of treatment with muCTLA4Ig on survival. Mice treated with muCTLA4Ig (closed circles); mice treated with L6 (open circles).

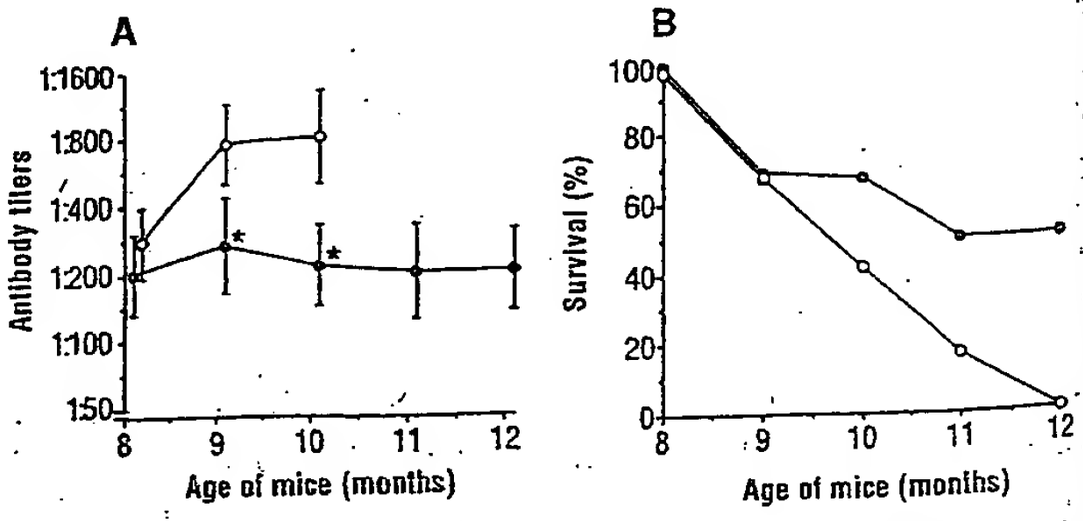

Fig. 2. (**A**) Effect of late treatment with muCTLA4Ig on the titer (geometric mean ± SEM) of antibodies to dsDNA. Forty female B/W mice were monitored until, at age 8 months, 40% of the original cohort had died from murine lupus and 50% of the surviving mice had proteinuria >100 mg/dl. At that time, two groups of the surviving mice (12 per group) began treatment with either muCTLA4Ig (closed circles) or L6 (open circles) for 4 months (50 μg, IP three times per week). Statistically significant differences between groups are denoted by asterisks. After 10 months of age, there were too few surviving control mice for further statistical comparison. Sera were analyzed as in Fig. 1A. (**B**) Effect of muCTLA4Ig on survival after initiation of treatment at age 8 months.

eatment, rather than prevention, of activ
itoimmunity, we tested muCTLA4Ig in th
te stages of clinical illness. A cohort of 40
male B/W mice was monitored until age 8
onths, by which time 40% of the mice had
ed from murine lupus. Then, at this ad-
nced stage of disease, we began treating the
maining mice with either muCTLA4Ig or
5 (50 μg, IP three times per week). At
itiation of therapy, the mean titer of anti-
odies to dsDNA was >1:200 (titer in nor-
al mouse serum was <1:50), and 50% of
ie mice had proteinuria >100 mg/dl. De-
ite the severity of the underlying illness,
eatment blocked further autoantibody pro-
uction (Fig. 2A) and prolonged life (Fig.
B). After 4 months of therapy, all of the
ontrol mice had died, whereas 50% of the
eated mice were still alive ($P < 0.02$, by χ^2
st). Thus, muCTLA4Ig can suppress an es-
iblished, pathological immune response.
his is the latest stage at which any interven-
on has been shown to be effective in B/W
iice.

Because selective inhibition of the B7-
D28 interaction can cause sustained anti-
en-specific T cell unresponsiveness in
itro (9, 12), it has been postulated that
TLA4Ig might cause antigen-specific T
ell tolerance in vivo. If this were true, it
ight be possible to achieve sustained in-
ibition of autoantigen-driven responses,
ven without specific knowledge of the in-
iting autoantigens. In our first experiment,
oduction of antibodies to dsDNA was
ppressed for 3 months after cessation of
herapy (Fig. 1A); it is possible, therefore,
hat this suppression is evidence for this
ypothesis. However, our studies were not

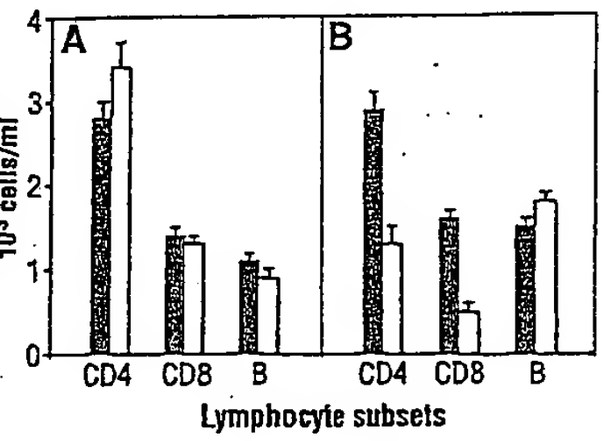

Fig. 3. Effect of treatment with muCTLA4Ig on peripheral blood lymphocyte subsets. Absolute lymphocyte counts from individual mice (12 mice per group) were obtained by means of an automated cell counter (Technicon Instruments, Tarrytown, New York). Peripheral blood lymphocytes subsets were then analyzed as described (2): A fluorescein-conjugated mAb to B220 (hybridoma RA3.6B2) detected B cells, mAb to CD4 (hybridoma GK1.5) detected $CD4^+$ T cells, and mAb to CD8 (hybridoma 53-6) detected $CD8^+$ T cells. The data shown here indicate the mean cell counts (± SEM) per milliliter of sera in mice treated with muCTLA4Ig (**A**) and in control mice treated with L6 (**B**) immediately before therapy at age 5 months (dark bars) and at the completion of therapy at age 9 months (white bars).

designed to establish conclusively whether in vivo tolerance was in fact achieved.

There are several mechanisms through which muCTLA4Ig might suppress murine lupus. It may exert its beneficial effects by binding to B7-related molecules on activated B cells and other APCs, preventing their interaction with CD28 and thus inhibiting the second signal for T cell activation. Alternatively, muCTLA4Ig may inhibit the function of activated T cells by blocking the interaction between CTLA-4 on activated T cells and B7 or a related ligand, B7-2 (*15–17*).

Beyond its effects on T cell function, muCTLA4Ig may suppress autoimmunity through direct effects on B cells. In particular, because murine IgG2a binds complement, muCTLA4Ig may effectively inhibit autoantibody production by depleting activated B cells. To assess this possibility, we monitored peripheral blood lymphocyte subsets (Fig. 3). These studies demonstrated that treatment with muCTLA4Ig did not significantly alter the absolute number of circulating B cells, suggesting that muCTLA4Ig did not suppress autoimmunity simply by the depletion of B cells. These findings are supported by studies in mice transgenic for CTLA4Ig that also imply that CTLA4Ig may not inhibit immune function by directly effecting B cells or by blocking the induction of T cell responses, but rather by blocking T cell effector functions, such as T cell help for B cells (*23*, *24*).

Treatment with muCTLA4Ig not only spared B cells, but it also prevented the progressive T cell lymphopenia that accompanies murine lupus in B/W mice (*2*). In control mice, there was an age-dependent decline in the absolute number of circulating T cells, which did not occur in mice treated with muCTLA4Ig (Fig. 3). This preservation of a stable lymphocyte profile throughout therapy most likely reflects amelioration of the underlying disease.

Attempts to suppress autoimmunity with biological agents have encountered two principal obstacles. First, treatment may have long-lasting adverse effects on the immune system, as has been seen in trials of antibodies to CD4 that caused prolonged depletion of $CD4^+$ T cells in people with rheumatoid arthritis (*3*). This observation raised questions about the long-term immune competence of treated individuals, as well as the feasibility of repeated courses of therapy. Our findings suggest that CTLA4Ig may circumvent this problem. Because muCTLA4Ig does not deplete lymphocytes, the effects of therapy on overall immune competence may be limited to the duration of therapy. Although this remains to be proven in B/W mice, studies in other strains indicate that, within a short time after cessation of therapy with muCTLA4Ig, the immune system is competent to mount normal immune responses (*20*). This may be particularly important in SLE because SLE is a disease with spontaneous remissions and relapses. Therefore, it is desirable to develop therapeutic strategies that might be used during periods of relapse without causing adverse effects that extend into periods of relative remission.

The second major obstacle to the use of biologic agents for chronic autoimmune diseases stems from the fact that many of these agents, such as mAbs or toxin conjugates, elicit a host immune response that can interfere with efficacy or cause toxicity (*25*). The use of CTLA4Ig may limit this problem. As a fusion product of two autologous proteins, CTLA4Ig appears to be poorly immunogenic. In B/W mice, for example, muCTLA4Ig elicited only a weak immune response that occurred after cessation of therapy, whereas human CTLA4Ig elicited an immediate and potent immune response (*26*). Consistent with these findings, human CTLA4Ig was cleared quickly from B/W mice and was ineffective against murine lupus, whereas muCTLA4Ig maintained high serum concentrations and succeeded in suppressing autoimmune disease.

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ORIGINAL ARTICLE

Treatment of Rheumatoid Arthritis by Selective Inhibition of T-Cell Activation with Fusion Protein CTLA4Ig

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ABSTRACT

BACKGROUND

Effective new therapies are needed for rheumatoid arthritis. Current therapies target the products of activated macrophages; however, T cells also have an important role in rheumatoid arthritis. A fusion protein — cytotoxic T-lymphocyte–associated antigen 4–IgG1 (CTLA4Ig) — is the first in a new class of drugs known as costimulation blockers being evaluated for the treatment of rheumatoid arthritis. CTLA4Ig binds to CD80 and CD86 on antigen-presenting cells, blocking the engagement of CD28 on T cells and preventing T-cell activation. A preliminary study showed that CTLA4Ig may be effective for the treatment of rheumatoid arthritis.

METHODS

We randomly assigned patients with active rheumatoid arthritis despite methotrexate therapy to receive 2 mg of CTLA4Ig per kilogram of body weight (105 patients), 10 mg of CTLA4Ig per kilogram (115 patients), or placebo (119 patients) for six months. All patients also received methotrexate therapy during the study. The clinical response was assessed at six months with use of the criteria of the American College of Rheumatology (ACR), which define the response according to its extent: 20 percent (ACR 20), 50 percent (ACR 50), or 70 percent (ACR 70). Additional end points included measures of the health-related quality of life.

RESULTS

Patients treated with 10 mg of CTLA4Ig per kilogram were more likely to have an ACR 20 than were patients who received placebo (60 percent vs. 35 percent, $P<0.001$). Significantly higher rates of ACR 50 and ACR 70 responses were seen in both CTLA4Ig groups than in the placebo group. The group given 10 mg of CTLA4Ig per kilogram had clinically meaningful and statistically significant improvements in all eight subscales of the Medical Outcomes 36-Item Short-Form General Health Survey. CTLA4Ig was well tolerated, with an overall safety profile similar to that of placebo.

CONCLUSIONS

In patients with active rheumatoid arthritis who were receiving methotrexate, treatment with CTLA4Ig significantly improved the signs and symptoms of rheumatoid arthritis and the health-related quality of life. CTLA4Ig is a promising new therapy for rheumatoid arthritis.

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RHEUMATOID ARTHRITIS IS A SYSTEMIC disease that causes progressive joint damage and disability.[1] The macrophage is an important pathogenic mediator in rheumatoid arthritis, and cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 are therapeutic targets. Drugs that block TNF-α decrease joint inflammation and slow radiographic progression.[2-8] However, since only approximately 40 percent of patients have an improvement of 50 percent, according to the criteria of the American College of Rheumatology (ACR), during treatment with TNF-α inhibitors, effective therapies directed against novel targets are needed.

Class II major-histocompatibility-complex (MHC) phenotype confers susceptibility to rheumatoid arthritis.[9] HLA-DR1 and DR4 are expressed in over 80 percent of white patients with rheumatoid arthritis.[10] Class II MHC molecules present antigens to CD4+ T cells, suggesting an important role of T cells in the pathogenesis of rheumatoid arthritis.

The rheumatoid synovium contains activated T cells, providing further support for the theory that T cells have an important role in rheumatoid arthritis.[11,12] Cells resembling monocytes and macrophages and dendritic cells are also present in the rheumatoid synovium. These antigen-presenting cells are activated and express both class II MHC and costimulatory molecules such as CD80 (B7-1) and CD86 (B7-2).[13-17] These observations suggest that synovial T cells, macrophages, dendritic cells, and B cells may have a direct role in the disease process.

T cells require at least two signals to become fully activated.[18,19] Signal 1 is antigen-specific and is delivered by engagement of the T-cell receptor with an MHC–peptide complex on an antigen-presenting cell. Signal 2 is delivered by the binding of a costimulatory receptor on T cells to a ligand on the antigen-presenting cell. A key costimulatory signal is provided by the interaction of CD28 on T cells with CD80 or CD86 on antigen-presenting cells.[20-22] In the presence of optimal T-cell–receptor and CD28 signals, T cells proliferate and produce cytokines that can activate other inflammatory cells, such as macrophages. With only a T-cell–receptor signal and no CD28 signal, T-cell activation is not optimal, and T cells may be rendered poorly responsive to otherwise optimal subsequent stimulation, or they may undergo apoptosis.[19]

Cytotoxic T-lymphocyte–associated antigen 4 (CTLA4) is expressed on the surface of T cells hours or days after they become activated. CTLA4 is the high-avidity receptor for both CD80 and CD86, binding approximately 500 to 2500 times as avidly to these ligands as to CD28.[23-25] CTLA4Ig is constructed by genetically fusing the external domain of human CTLA4 to the heavy-chain constant region of human IgG1. CTLA4Ig binds both CD80 and CD86 on antigen-presenting cells, thereby preventing these molecules from engaging CD28 on T cells. By blocking the engagement of CD28, CTLA4Ig prevents the delivery of the second costimulatory signal that is required for optimal activation of T cells. Blocking the second signal is a novel therapeutic concept. Preclinical studies demonstrated the efficacy of CTLA4Ig in many animal models of autoimmune disease[26,27] and allograft rejection.[28]

In a three-month pilot study in which patients with rheumatoid arthritis were given 0.5, 2, or 10 mg of CTLA4Ig per kilogram of body weight as monotherapy on days 1, 15, 29, and 57, 53 percent of patients who received the dose of 10 mg per kilogram had a 20 percent improvement (an ACR 20 response) after 85 days and 16 percent had a 50 percent improvement (an ACR 50 response), according to the ACR criteria.[29] Here, we report the results of a six-month, double-blind, randomized, placebo-controlled investigation of the effectiveness of CTLA4Ig therapy in patients with rheumatoid arthritis who had an inadequate response to methotrexate.

METHODS

PATIENTS

The study population consisted of patients 18 to 65 years of age who met the ACR criteria for rheumatoid arthritis and were in functional class I, II, or III.[30] Entry requirements included active disease, characterized by 10 or more swollen joints, 12 or more tender joints, and C-reactive protein levels of at least 1 mg per deciliter (upper limit of the normal range, 0.4). Patients had to have been treated with methotrexate (10 to 30 mg weekly) for at least 6 months and to have received a stable dose for 28 days before enrollment. All patients continued to receive methotrexate. All other disease-modifying antirheumatic drugs were discontinued. Leflunomide and infliximab were discontinued at least 60 days before enrollment, and other disease-modifying antirheumatic drugs were discontinued at least 28 days before enrollment. Stable low-dose corticosteroids ($\leq$10 mg per day) and nonsteroidal antiinflammatory drugs were permitted. Women who were nursing

or pregnant were excluded. Patients were enrolled in the study between December 11, 2000, and December 11, 2001.

STUDY PROTOCOL

This was a six-month randomized, double-blind, placebo-controlled study to compare the safety, efficacy, and immunogenicity of 2 mg or 10 mg of CTLA4Ig per kilogram with those of placebo in patients with active rheumatoid arthritis. The study sponsor was involved in the design of the study, collection of the data, and analysis of the data. The academic investigators had access to the data and were responsible for interpreting the data. The protocol was approved by the appropriate international regulatory boards and the human-research committees at each participating center. Written informed consent was obtained from all patients before they underwent randomization or any study-related procedures. A central randomization procedure was used. To ensure that the treatment groups were balanced at each site, patients were randomly assigned with use of a permuted-block size of 6. CTLA4Ig or placebo was infused intravenously over a 30-minute period on days 1, 15, and 30 and monthly thereafter for a total of six months.

EFFICACY MEASUREMENTS

The primary efficacy variable was the percentage of patients who had a 20 percent improvement according to ACR criteria (an ACR 20 response) at six months.[31] The ACR criteria assess 68 joints for tenderness and 66 joints for swelling. An ACR 20 response indicates a decrease of at least 20 percent in both the number of tender joints and the number of swollen joints, as well as a 20 percent improvement in at least three of the following: the patient's global assessment of disease status, the patient's assessment of pain, the patient's assessment of physical function (measured with use of the Modified Stanford Health Assessment Questionnaire), the physician's global assessment of disease status, and the C-reactive protein level. Secondary outcome measures were 50 percent improvement and 70 percent improvement according to ACR criteria (an ACR 50 response and an ACR 70 response, respectively). The ACR response was assessed on days 1, 15, and 30 and then monthly. Assessments were performed by rheumatologists or trained professional staff members who were unaware of patients' treatment assignments and were not involved in the infusion of CTLA4Ig or placebo.

Health-related quality of life was assessed at base line, 90 days, and 180 days with use of the Medical Outcomes Study 36-Item Short-Form General Health Survey (SF-36).[32,33] The SF-36 consists of 36 items, 35 of which are aggregated to evaluate eight dimensions of health: physical function, pain, general and mental health, vitality, social function, and physical and emotional health. Scores on the eight subscales were aggregated to derive the physical-

Table 1. Base-Line Characteristics of the Patients.*

Characteristic	Placebo + Methotrexate (N=119)	CTLA4Ig, 2 mg/kg, + Methotrexate (N=105)	CTLA4Ig, 10 mg/kg, + Methotrexate (N=115)
Age (yr)			
Mean	54.7	54.4	55.8
Range	23–80	23–80	17–83
Weight (kg)			
Mean	79.9	78.7	77.8
Range	44–140	48–186	40–144
Female sex (%)	66	63	75
White race (%)	87	87	87
Duration of disease (yr)	8.9±8.3	9.7±8.1	9.7±9.8
Methotrexate dose (mg/wk)	15.8±4.1	15.8±4.5	15.0±4.4
Mean duration of methotrexate therapy (yr)	2.9±3.5	2.6±3.0	2.5±2.7
Previous treatment with anti-TNF drug (%)	2.6	5.7	2.6
Joints (no.)†			
Tender	29.2±13.0	28.2±12.0	30.8±12.2
Swollen	21.8±8.8	20.2±8.9	21.3±8.4
Pain score‡	65.2±22.1	64.5±22.3	62.1±21.4
Physical-function score§	1.0±0.6	1.0±0.5	1.0±0.5
Global assessment score‡			
Patient	62.8±21.6	59.4±23.7	60.1±20.7
Physician	63.3±15.5	61.0±16.7	62.1±14.8
Positive for rheumatoid factor (%)	90	90	99
Erosive disease (%)	100	100	100
C-reactive protein (mg/dl)	3.2±3.2	3.2±2.6	2.9±2.8
SF-36 score¶			
Physical component	32.3±7.7	30.8±8.5	31.3±8.5
Mental component	41.9±11.0	43.1±11.0	44.5±10.5

* Plus–minus values are means ±SD. TNF denotes tumor necrosis factor.
† Sixty-eight joints were assessed for tenderness, and 66 were assessed for swelling.
‡ A 100-mm visual-analogue scale was used in which higher values indicated more severe abnormalities.
§ The Modified Stanford Health Assessment Questionnaire was used. Scores can range from 0 to 3, with higher scores indicating greater disease activity.
¶ Scores on the Medical Outcomes Study 36-Item Short-Form General Health Survey (SF-36) were standardized on the basis of a mean score of 50±10 in the general U.S. population. Higher scores indicate a better quality of life.

component summary score and the mental-component summary score. The eight subscales, physical-component summary, and mental-component summary were scored with use of norm-based methods that standardize the scores to a mean (±SD) of 50±10 on the basis of an assessment of the general U.S. population of persons without chronic conditions.[34] Scores on each subscale range from 0 to 10, and the summary scores range from 0 to 100, with higher scores indicating better health. Absolute differences of three or more in both the subscale scores and summary scores were considered clinically meaningful.[35,36]

SAFETY ASSESSMENTS

Patients were asked about adverse events at each visit, and the investigator assessed the severity of any reported event and its relation to the study medication. A data and safety monitoring board supervised the overall safety assessment in an unblinded fashion.

IMMUNOGENICITY TESTING

Serum samples were obtained for the measurement of drug-specific antibodies on days 1, 30, 90, and 180. Formation of specific antibody against the whole molecule (CTLA4Ig) and against the CTLA4 portion alone were evaluated separately according to previously described methods.[29] Results were expressed as the end-point titer, defined as the reciprocal of the interpolated dilution with an absorbance value equal to five times the mean absorbance background value. Seroconversion was defined by an increase of at least two serial dilutions (by a factor of nine) relative to the predose value.

STATISTICAL ANALYSIS

A sample of 107 patients per treatment group was determined to yield 94 percent power at the 5 percent level (two-sided) to detect an absolute difference of 25 percent between the group given 10 mg of CTLA4Ig per kilogram and the group given pla-

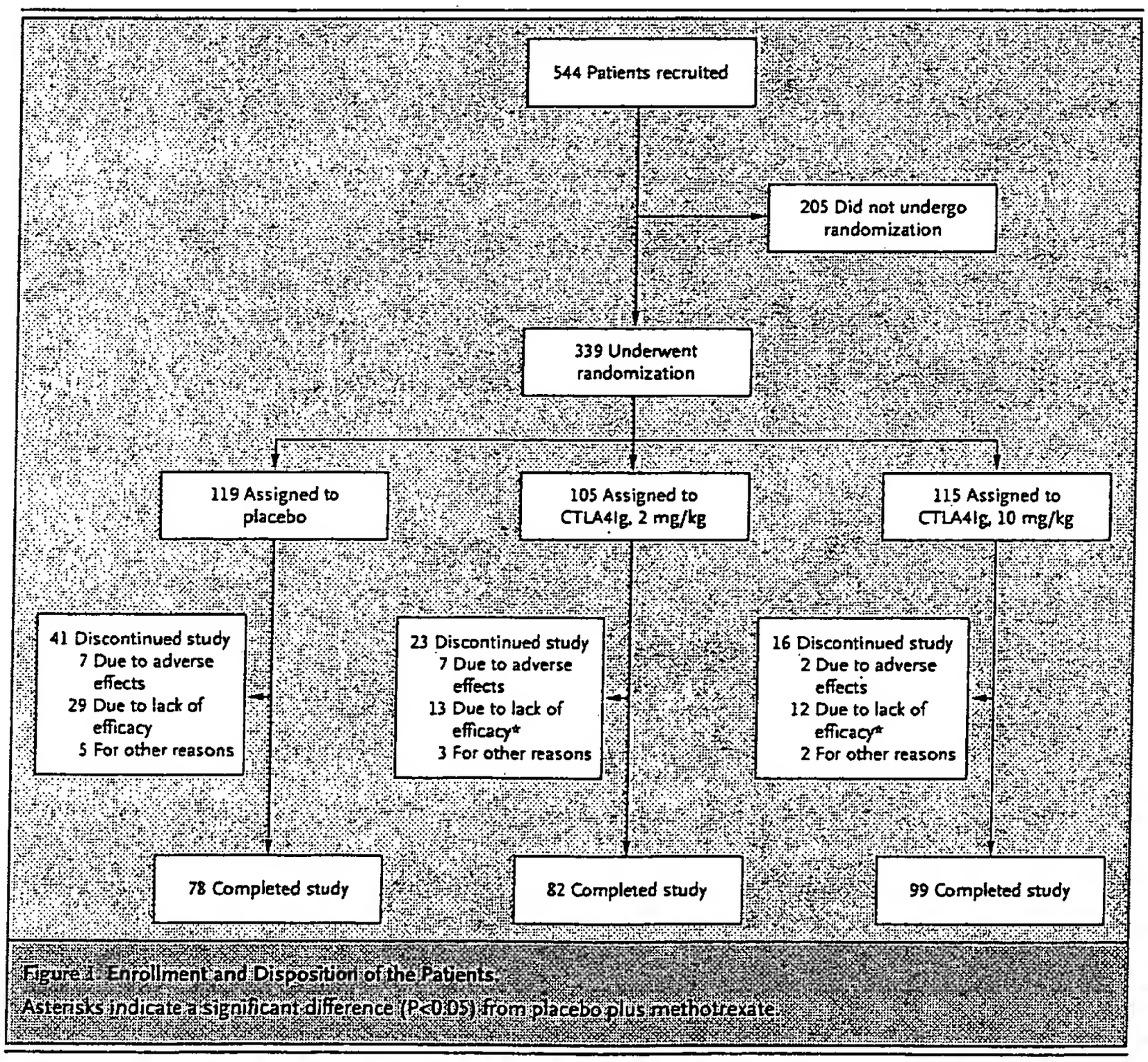

Figure 1. Enrollment and Disposition of the Patients.
Asterisks indicate a significant difference ($P<0.05$) from placebo plus methotrexate.

cebo plus methotrexate, on the basis of an expected ACR 20 response rate at six months of 25 percent in the placebo group and a dropout rate of 15 percent in each treatment group. A closed testing procedure based on an ordered analysis of variance[37] was established for hypothesis testing: if there was a significant difference in the rates of ACR 20 responses between the group given 10 mg of CTLA4Ig per kilogram and the placebo group with use of a chi-square test, then we compared the group given 2 mg of CTLA4Ig per kilogram with the placebo group. This testing strategy was also used to identify differences in the rates of ACR 50 and ACR 70 responses.

Descriptive statistics were used to compare the demographic and base-line characteristics of the patients in the three treatment groups. The efficacy analyses included all patients who received at least one dose of study medication. To account for missing data in the assessment of the ACR responses in the primary, prespecified analysis, we considered patients who discontinued the study because of worsening disease not to have had a response, and we carried forward the values obtained at the last assessment for patients who discontinued the study for any other reason. Thus, all patients were assessed for an ACR response. When assessing the change from base line in the health-related quality of life and the individual components of the ACR response in patients who discontinued the study for any reason, we used the values obtained at the last assessment and carried them forward. A secondary analysis was performed in which all patients who discontinued the study for any reason were classified as having had no response.

Fisher's exact tests were used to compare the incidence of adverse events in the CTLA4Ig groups and the placebo group. For other end points, analysis of covariance (adjusted for base-line values) with linear contrasts was used for continuous variables and chi-square tests were used for proportions. All statistical tests were two-sided and conducted at the 5 percent level.

RESULTS

CHARACTERISTICS OF THE PATIENTS

Study medication was administered to 339 patients: 119 patients were randomly assigned to receive placebo plus methotrexate, 105 to receive 2 mg of CTLA4Ig per kilogram plus methotrexate, and 115 patients to receive 10 mg of CTLA4Ig per kilogram plus methotrexate. The demographic and base-line clinical characteristics were similar among the treatment groups (Table 1). Despite concurrent treatment with methotrexate, patients had a high degree of base-line disease activity on the basis of the numbers of swollen and tender joints.

A total of 259 patients completed six months of treatment (Fig. 1). More patients in the placebo group discontinued the study than in either of the CTLA4Ig groups. The most common reason for discontinuation was lack of efficacy as indicated by worsening arthritis.

CLINICAL EFFICACY

The percentage of patients who had an ACR 20 response at six months was significantly higher in the group given 10 mg of CTLA4Ig per kilogram than in the placebo group (Fig. 2 and Table 2). There was no significant difference in the rate of ACR 20 responses at six months between the group given 2 mg of CTLA4Ig per kilogram and the placebo group ($P=0.31$). ACR 20 responses in the group given 10 mg of CTLA4Ig per kilogram were significantly higher than those in the placebo group from month 2 through month 6 (Fig. 2).

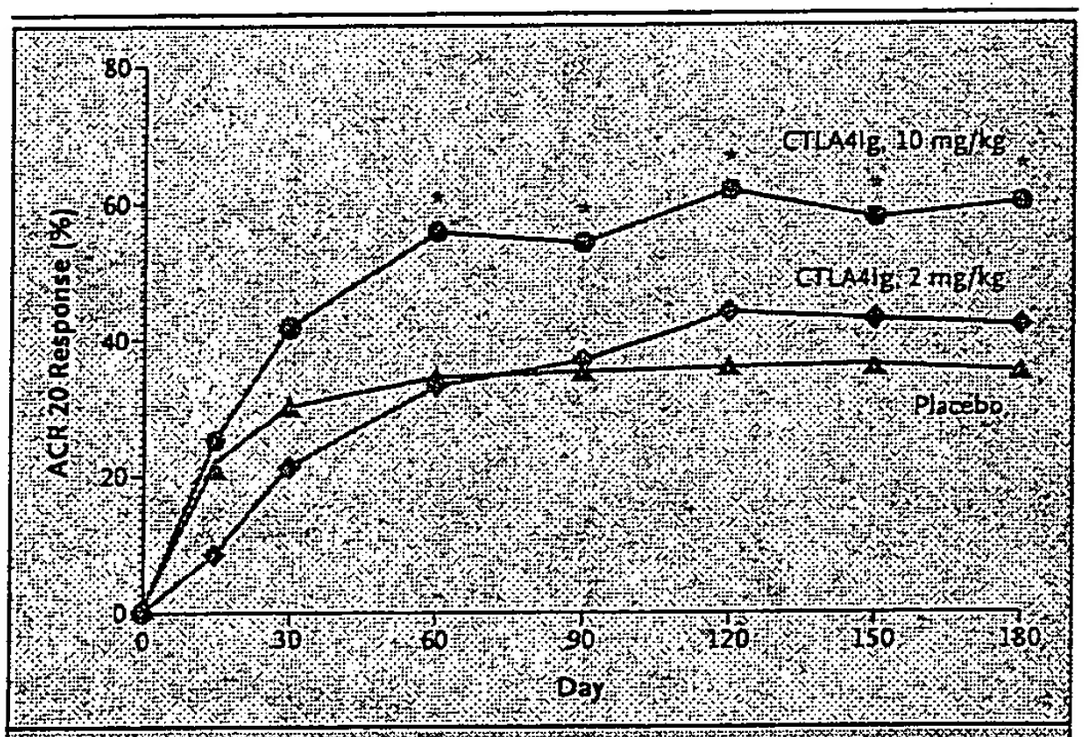

Figure 2. Clinical Efficacy of CTLA4Ig.
A clinical response was defined according to the American College of Rheumatology (ACR) definition of a 20 percent improvement (ACR 20), indicating a decrease of at least 20 percent in the number of both tender joints and swollen joints, along with a 20 percent improvement in three of the following: the patient's global assessment of disease status, the patient's assessment of pain, the patient's estimate of physical function (measured with use of the Modified Stanford Health Assessment Questionnaire), the physician's global assessment of disease status, and the serum C-reactive protein level. At each study visit, measurements were obtained before any treatment was administered. Asterisks indicate a significant difference ($P<0.001$) between the group given 10 mg of CTLA4Ig per kilogram and the placebo group.

Table 2. Efficacy at Six Months.*

Variable	Placebo + Methotrexate (N=119)	CTLA4Ig, 2 mg/kg, + Methotrexate (N=105)	CTLA4Ig, 10 mg/kg, + Methotrexate (N=115)
		percent	
ACR response rate†			
ACR 20	35.3	41.9	60.0‡
ACR 50	11.8	22.9§	36.5‡
ACR 70	1.7	10.5§	16.5‡
Mean change from base line in individual ACR components¶			
Tender joints	32.1	43.3	59.9§
Swollen joints	33.4	45.1§	54.9§
Pain	8.4	22.7§	46.4§
Physical function	14.1	17.3	41.5§
Patient's global assessment	17.6	9.6	40.8§
Physician's global assessment	25.6	38.6§	52.0§
C-reactive protein level	−23.6	16.2§	31.5§

* A clinical response was defined according to the American College of Rheumatology (ACR) definition of a 20 percent improvement (ACR 20), indicating a decrease of at least 20 percent in the number of both tender joints and swollen joints, along with a 20 percent improvement in at least three of the following: the patient's global assessment of disease status, the patient's assessment of pain, the patient's estimate of function (measured with use of the Modified Stanford Health Assessment Questionnaire), the physician's global assessment of disease status, and the serum C-reactive protein level. The percentages of patients with an improvement of 50 percent (ACR 50) and 70 percent (ACR 70), according to the ACR criteria, were assessed in a similar manner.
† Patients who discontinued the study because of worsening disease were considered to have had no response; for those who discontinued the study for other reasons the values for the last efficacy observation were carried forward.
‡ $P<0.001$ for the comparison with the group given placebo plus methotrexate.
§ $P<0.05$ for the comparison with the group given placebo plus methotrexate.
¶ Values were carried forward from the last efficacy observation.

The rates of ACR 50 and ACR 70 responses at six months were significantly higher in both CTLA4Ig groups than in the placebo group (Table 2). As compared with the patients in the placebo group, patients who received 10 mg of CTLA4Ig per kilogram also had significant improvements in all clinical components of the ACR response criteria (Table 2).

In a secondary analysis, patients who discontinued the study for any reason were classified as having had no response. In this analysis, the rate of ACR 20 responses at six months was significantly higher in the group given 10 mg of CTLA4Ig per kilogram than in the placebo group (57.4 percent vs. 31.1 percent, $P<0.001$). The rate of ACR 20 responses in the group given 2 mg of CTLA4Ig per kilogram was 39 percent and did not differ significantly from that in the placebo group ($P=0.21$). The rates of ACR 50 responses were 35.7 percent in the group given 10 mg of CTLA4Ig per kilogram and 22.9 percent in the group given 2 mg of CTLA4Ig per kilogram, as compared with 10.1 percent in the placebo group ($P<0.001$ and $P=0.009$, respectively). The rates of ACR 70 responses were the same as those in the primary analysis.

Patients in the group given 10 mg of CTLA4Ig per kilogram had clinically meaningful and significant improvements from base-line scores in the scores on all eight subscales and both summary scores of the SF-36, with the greatest effect in the physical-health, pain, vitality, and social-function domains (Fig. 3). All improvements were significantly greater than those in the placebo group ($P<0.05$). For patients treated with 2 mg of CTLA4Ig per kilogram, improvements from base-line values were significant for all domains except mental health but did not differ significantly from those in the placebo group.

SAFETY

CTLA4Ig was well tolerated, and no deaths, cancers, or opportunistic infections were reported by CTLA4Ig-treated patients after six months of treatment. In general, adverse events were reported at a similar or lower rate in the CTLA4Ig groups than in the placebo group. The most frequently reported adverse event was headache, followed in decreasing order by upper respiratory tract infection, musculoskeletal pain, and nausea and vomiting (Table 3).

Fewer serious adverse events were reported in the group given 10 mg of CTLA4Ig per kilogram than in the group given 2 mg of CTLA4Ig per kilogram or the placebo group (Table 3). None of the serious adverse events in the group given 10 mg of CTLA4Ig per kilogram were considered to be related to the study drug. One patient in the group given 2 mg of CTLA4Ig per kilogram was hospitalized for cellulitis of the left foot. No other serious infections were reported. The rate of discontinuation because of adverse events was lower in the group given 10 mg of CTLA4Ig per kilogram (1.7 percent) than in the group given 2 mg of CTLA4Ig per kilogram (6.7 percent) or the placebo group (5.9 percent).

IMMUNOGENICITY TESTING

Most patients had preexisting antibodies against CTLA4Ig. No patient in either of the CTLA4Ig

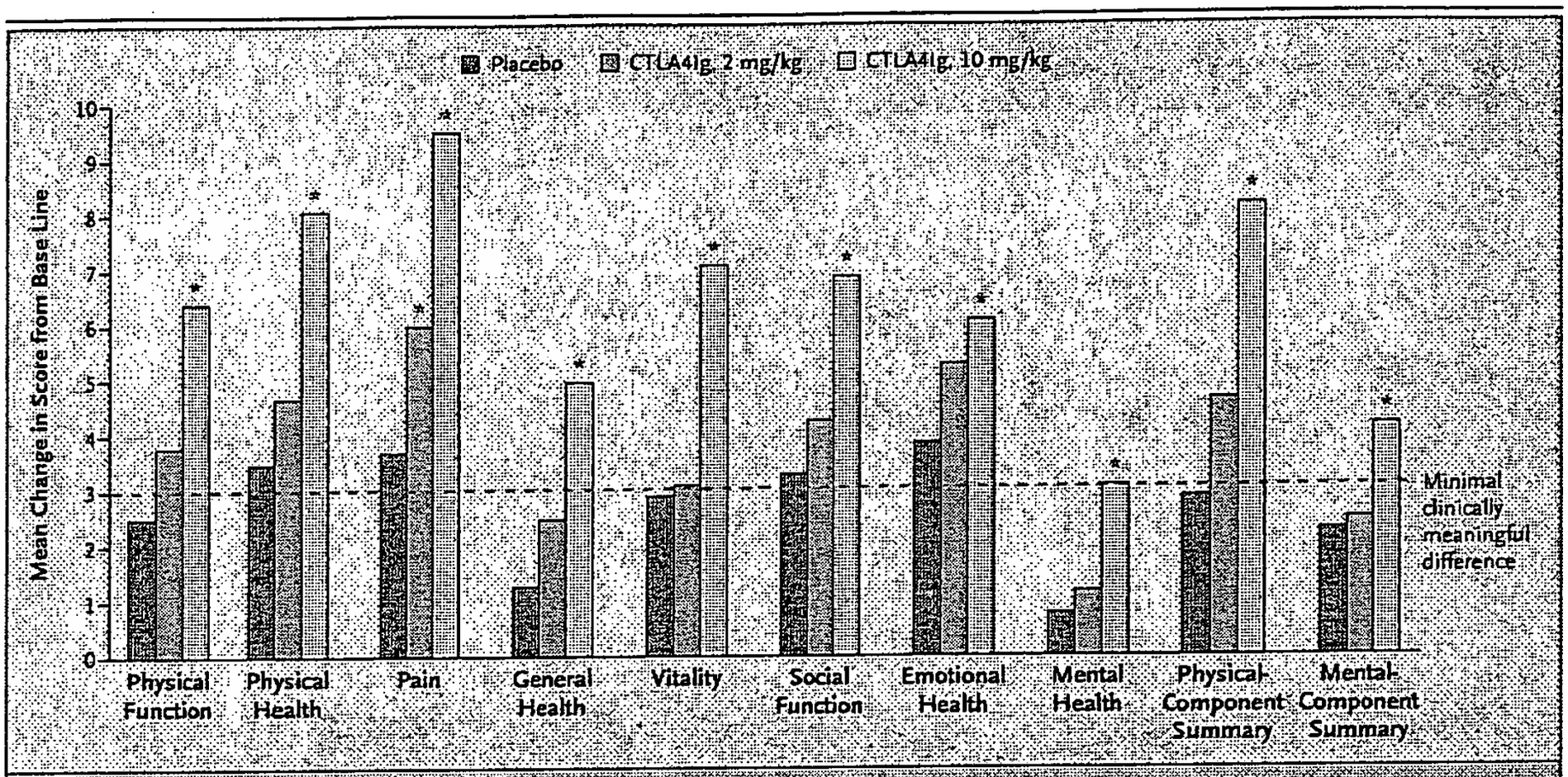

Figure 3. Effect of CTLA4Ig on the Health-Related Quality of Life.
Health-related quality of life was assessed with use of the Medical Outcomes Study 36-Item Short-Form General Health Survey (SF-36).[32,33] Scores on the eight subscales of the SF-36 were aggregated to derive the physical-component summary score and the mental-component summary score. The eight subscales, physical-component summary, and mental-component summary were scored with use of norm-based methods that standardize the scores to a mean (±SD) of 50±10 on the basis of an assessment of the general U.S. population of persons without chronic conditions.[34] Scores on each subscale range from 0 to 10, and the summary scores range from 0 to 100, with higher scores indicating better health. Asterisks indicate a significant difference ($P<0.05$) for the comparison with the placebo group with use of an analysis of covariance model with the base-line value as a covariate. Values were carried forward from the last efficacy observation. Changes from base line were also significant for each subscale score and for the summary scores in the 10-mg CTLA4Ig group.

groups had evidence of seroconversion for CTLA4Ig-specific antibodies during the six-month study period. Seroconversion for CTLA4-specific antibodies was detected in one patient in the group given 10 mg of CTLA4Ig per kilogram (the end-point titer increased from less than 10 at base line to 92 at one month [the last sample collected]) and in one patient in the group given 2 mg of CTLA4Ig per kilogram (the end-point titer increased from less than 10 at base line to 148 at six months).

DISCUSSION

The goal of clinical management of rheumatoid arthritis has been to avert disease progression through treatment with disease-modifying antirheumatic drugs such as methotrexate, sulfasalazine, leflunomide, and hydroxychloroquine. More recently, biologic agents targeting specific inflammatory cytokines such as TNF-α and interleukin-1 have been prescribed for patients with an inadequate response to methotrexate. Even with the use of these newer therapies, many patients do not have a satisfactory response.

CTLA4Ig is the first in a new class of drugs for the treatment of rheumatoid arthritis known as costimulation blockers. Current biologic agents specifically block the activity of single cytokines produced predominantly by macrophages. CTLA4Ig acts earlier in the inflammatory cascade and directly inhibits the activation of T cells and the secondary activation of other important cells, such as macrophages and B cells. Recently, Grohmann et al.[38] demonstrated that CTLA4Ig has a direct inhibitory effect on dendritic cells and macrophages. The binding of CTLA4Ig to CD80 and CD86 appears to lead to the production of indoleamine-2,3-dioxygenase by antigen-presenting cells, which is associated with down-regulation of the inflammatory responses of T cells, dendritic cells, and macrophages.[39,40]

In this six-month trial, CTLA4Ig therapy induced dose-related improvements in the signs and symptoms of rheumatoid arthritis and in physical function. The magnitude of the ACR 20, ACR 50, and

Table 3. Adverse Events.

Adverse Event	Placebo + Methotrexate (N=119)	CTLA4Ig, 2 mg/kg, + Methotrexate (N=105)	CTLA4Ig, 10 mg/kg, + Methotrexate (N=115)
	number (percent)		
Death	0	0	0
Serious adverse events			
Total	12 (10.1)	12 (11.4)	3 (2.6)*
Related to study drug	1 (0.8)	4 (3.8)	0
Most frequent adverse events†			
Headache	15 (12.6)	15 (14.3)	12 (10.4)
Upper respiratory tract infection	12 (10.1)	13 (12.4)	15 (13.0)
Musculoskeletal pain	15 (12.6)	15 (14.3)	8 (7.0)
Nausea and vomiting	14 (11.8)	7 (6.7)	16 (13.9)
Fatigue	13 (10.9)	10 (9.5)	6 (5.2)
Cough	10 (8.4)	6 (5.7)	12 (10.4)
Diarrhea	7 (5.9)	7 (6.7)	11 (9.6)
Pharyngitis	7 (5.9)	5 (4.8)	12 (10.4)

* P=0.03 for the comparison with the group given placebo plus methotrexate.
† Rheumatoid arthritis was not included.

ACR 70 responses after treatment with 10 mg of CTLA4Ig per kilogram (60.0 percent, 36.5 percent, and 16.5 percent, respectively) was similar to that in patients who received methotrexate after treatment with 10 mg of infliximab per kilogram every four weeks (ACR 20, 58 percent; ACR 50, 26 percent; and ACR 70, 11 percent).[41] Furthermore, the combination of 10 mg of CTLA4Ig per kilogram and methotrexate resulted in clinically meaningful and significant improvements over base-line scores on all eight subscales of the SF-36.

CTLA4Ig was safe and well tolerated, and the rate of discontinuation because of adverse events was no higher than that in the placebo group. In addition, no clinically significant antibody response to CTLA4Ig was detected in either active-treatment group.

In the analysis in which all patients who discontinued the study were considered not to have had a response, the ACR responses remained significant. The low rates of serious adverse effects and discontinuation owing to adverse events, especially with the dose of CTLA4Ig of 10 mg per kilogram, provides further support for its use in the treatment of rheumatoid arthritis. However, longer-term observation of the safety and efficacy of CTLA4Ig in combination with methotrexate, especially with regard to infection, is needed to confirm and extend these encouraging findings.

We found that the combination of CTLA4Ig and methotrexate improved the signs and symptoms of disease, physical function, and quality of life in patients who had active rheumatoid arthritis despite ongoing methotrexate therapy. Clinical responses were dose-dependent. Both the 2 mg per kilogram dose and the 10 mg per kilogram dose of CTLA4Ig were well tolerated, with no antibody response to the fusion protein detected. These data underscore the value of costimulation blockade in the treatment of rheumatoid arthritis. The potential use of CTLA4Ig in the treatment of rheumatoid arthritis and other autoimmune disorders requires further investigation.

Drs. Kremer and Emery report having received grant support from Bristol-Myers Squibb and having served as paid consultants to the company. Drs. Alten, Leon, Dougados, and Moreland report having served as paid consultants to Bristol-Myers Squibb. Drs. Nuamah, Williams, Becker, and Hagerty are employees of Bristol-Myers Squibb.

APPENDIX

In addition to the authors, the following investigators also participated in the study: A. Bankhurst (Albuquerque, N.M.), A. Beaulieu (Sainte-Foy, Que., Canada), R. Bernstein (Manchester, United Kingdom), C. Birbara (Worcester, Mass.), B. Bockow (Seattle), L. Bridges, Jr. (Birmingham, Ala.), S. Brighton (Pretoria, South Africa), W. Chase (Austin, Tex.), B. Combe (Montpellier, France), B. Diamond (Bronx, N.Y.), G.S. Dolan (Long Beach, Calif.), P. Dura (Endwell, N.Y.), P. Durez (Brussels, Belgium), R. Fleishmann (Dallas), S. Hall (Malvern, Victoria, Australia), A. Hammond (Maidstone, Kent, United Kingdom), P. Hanrahan (South Perth, Western Australia, Australia), B. Haraoui (Montreal), B. Hazleman (Cambridge, United Kingdom), G. Hein (Jena, Germany), R. Honsinger (Los Alamos, N.M.), R. Katz (Chicago), E. Keystone (Toronto), M. Khraishi (St. Johns, Newf., Canada), A. Kivitz (Duncansville, Pa.), S. Klein (Cumberland, Md.), R. Leff (Duluth, Minn.), P. Liang (Sherbrooke, Que., Canada), R. Lies (Wichita, Kans.), J.M. Cocco (Buenos Aires, Argentina), R. McKendry (Ottawa, Ont., Canada), B. Miskin (West Palm Beach, Fla.), R. Moidel (Sellersville, Pa.), M. Molloy (Wilton, Ireland), J. Peller (Rome, Ga.), H. Peter (Freiburg, Germany), D. Pierangelo (Springfield, Mass.), K. Pile (Woodville, South Australia, Australia), A. Rosen (Largo, Fla.), C. Saadeh (Amarillo, Tex.), R. Salach (Titusville, Fla.), J. Sany (Montpellier, France), W. Shergy (Huntsville, Ala.), J. Sibilia (Strasbourg, France), W. St. Clair (Durham, N.C.), E. Tindall (Portland, Oreg.), P.L.C.M. Van Riel (Nijmegen, the Netherlands), F. vas den Bosch (Ghent, Belgium), A. Weaver (Lincoln, Nebr.), and L. Williame (Antwerp, Belgium).

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Induction of Alloantigen-specific Hyporesponsiveness in Human T Lymphocytes by Blocking Interaction of CD28 with Its Natural Ligand B7/BB1

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Summary

The specificity of T lymphocyte activation is determined by engagement of the T cell receptor (TCR) by peptide/major histocompatibility complexes expressed on the antigen-presenting cell (APC). Lacking costimulation by accessory molecules on the APC, T cell proliferation does not occur and unresponsiveness to subsequent antigenic stimulus is induced. The B7/BB1 receptor on APCs binds CD28 and CTLA-4 on T cells, and provides a costimulus for T cell proliferation. Here, we show that prolonged, specific T cell hyporesponsiveness to antigenic restimulation is achieved by blocking the interaction between CD28 and B7/BB1 in human mixed leukocyte culture (MLC). Secondary T cell proliferative responses to specific alloantigen were inhibited by addition to the primary culture of monovalent Fab fragments of anti-CD28 monoclonal antibody (mAb) 9.3, which block interaction of CD28 with B7/BB1 without activating T cells. Hyporesponsiveness was also induced in MLC by CTLA4Ig, a chimeric immunoglobulin fusion protein incorporating the extracellular domain of CTLA-4 with high binding avidity for B7/BB1. Cells previously primed could also be made hyporesponsive, if exposed to alloantigen in the presence of CTLA4Ig. Maximal hyporesponsiveness was achieved in MLC after 2 d of incubation with CTLA4Ig, and was maintained for at least 27 d after removal of CTLA4Ig. Accumulation of interleukin 2 (IL-2) and interferon γ but not IL-4 mRNA was blocked by CTLA4Ig in T cells stimulated by alloantigen. Antigen-specific responses could be restored by addition of exogenous IL-2 at the time of the secondary stimulation. Addition to primary cultures of the intact bivalent anti-CD28 mAb 9.3, or B7/BB1$^+$ transfected CHO cells or exogenous IL-2, abrogated induction of hyporesponsiveness by CTLA4Ig. These data indicate that interaction of CD28 with B7/BB1 during TCR engagement with antigen is required to maintain T cell competence and that blocking such interaction can result in a state of T cell hyporesponsiveness.

Effective presentation of antigen to T cells requires a complex series of events to initiate the immune response. In addition to processing and presenting antigenic peptides in the context of MHC molecules to specific TCRs, APCs must provide one or more costimulatory signal(s) to fully activate T cells, and induce IL-2 release and DNA synthesis (1–7). In the absence of costimulatory signals, T cells presented with antigen may enter a state of anergy characterized by the failure to activate the IL-2 gene in response to further antigenic stimulation (4). In certain instances, lack of costimulation may lead to activation-driven cell death (8). Binding of surface receptors on T cells to their natural ligands, such as CD2 to LFA-3 (9), CD4 to MHC class II (9), LFA-1 to intercellular adhesion molecule 1 (ICAM-1) or ICAM-2 (10), and CD28 to B7/BB1 (11) have been implicated in facilitating T cell–APC interactions and inducing T cell activation. CD28 signaling stimulates cytokine production by T cells, by regulating gene transcription and also by stabilizing mRNAs (12–16). Binding of CD28 to the B7/BB1 counter receptor costimulates IL-2 mRNA accumulation and T cell proliferation (17–20). CD28-mediated signaling prevents induction of anergy in murine T cell clones (21).

CTLA-4, a molecule homologous to CD28 originally identified by screening of a murine cytolytic T cell cDNA library (22), also binds to B7/BB1 (23). Studies of the binding properties of CTLA-4 and B7/BB1 were facilitated by con-

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struction of a soluble fusion protein consisting of the extracellular domain of CTLA-4 and an IgG γ1 chain (23). CTLA4Ig has a high avidity for the B7/BB1 molecule (K_d ~12 nM) and is a potent inhibitor of immune responses in vitro and in vivo (23–25).

In this study, we have investigated the role of CD28 interactions with B7/BB1 in providing the costimulation necessary to maintain proliferative competence of human T cells. We have found that blocking the interaction of CD28 with B7/BB1 either by anti-CD28 mAb 9.3 Fab fragments or by CTLA4Ig when T cells are presented with alloantigen in a mixed leukocyte culture (MLC)[1] leads to sustained T cell hyporesponsiveness to the specific alloantigen.

Materials and Methods

Ig Fusion Proteins, mAbs, and Transfected Cell Lines. CTLA4Ig was produced by CHO cells transfected with the CTLA4Ig cDNA expression construct and was purified as described previously (23). Purified human mouse chimeric mAb L6 was a gift of Ingegerd and Karl Erik Hellstrom (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Murine mAbs 9.3 (anti-CD28, IgG2a), BB1 (anti-B7/BB1 antigen, IgM), 9E8 (anti-p15E, IgG2a), and T11D7, (anti-Thy1.1, IgM, kind gift of Irwin Bernstein, Fred Hutchinson Cancer Research Center) have been described previously and were purified from ascites before use (26–28). Preparation of Fab fragment of mAb 9.3 has been described previously (29). B7$^+$ CHO cells have been previously described (23) and CD5$^+$ CHO cells were constructed as described (19, 23) using an expressible CD5 cDNA provided by Dr. A. Aruffo (Bristol-Myers Squibb Pharmaceutical Research Institute).

Primary MLC. PBMC were prepared by density gradient centrifugation on Ficoll-Hypaque. The cells were resuspended in medium containing RPMI 1640, 25 mM Hepes, 1 U/ml penicillin, 1 μg/ml streptomycin, and 15% pooled human serum that had been heat inactivated at 56°C for 30 min. As indicated for certain experiments, T cell subsets were purified by negative selection using complement-dependent lysis and panning (30). Responders and stimulators were unrelated individuals chosen so that there was at least one HLA class I and one HLA-DR antigen mismatched within each pair. 5×10^4 responder cells were mixed with 5×10^4 irradiated stimulator cells (3,000 rad) in round-bottomed 96-well plates. These were incubated at 37°C in a 5% CO_2 atmosphere. Assays were performed in triplicate. Cultures were pulsed with one μCi of [^{3}H]thymidine 18 h before harvesting. 10 replicate plates were set up and one was harvested each day for 10 consecutive d. Data are reported as mean cpm of the three replicates. In selected experiments, readings were taken on day 6 of the MLC.

Restimulation Assays. 10^7 PBMC from one individual were primed with an equivalent number of irradiated (3,000 rad) PBMC from another HLA class I- and II-incompatible individual in 25 cm^2 flasks, using identical culture conditions as for primary MLC carried out in 96-well plates. For blocking experiments, cells were cultured for 7 d in the presence of human Ig fusion proteins CTLA4Ig or human–mouse chimeric mAb L6, used as control. Then cells were washed three times, recultured in medium without Ig for an additional 3 d, harvested on day 10, and then restimulated. Primed cells were restimulated with fresh stimulator cells from the original donor or from an unrelated donor. The two donors did not share HLA-DR, DQ, or DP antigens. In experiments of tertiary stimulation, a secondary culture was carried out in flask, as in the first. As indicated in certain experiments, alloantigen-primed CD4$^+$ T cell lines were generated by stimulation with cells from an EBV-transformed B line from an unrelated donor. For the assay, 2×10^4 primed responders and 5×10^4 irradiated stimulators were incubated in 96-well round-bottomed wells in medium without any Ig fusion protein. Assays were performed as detailed for primary MLC.

Generation of CTL. Fresh PBMC or primed cells were tested for CTL precursor activity by priming in MLC. Responder cells (10^7) either fresh or primed as specified for each experiment, and irradiated stimulators (10^7), were cultured for 6 d, harvested, washed twice, and tested for cytolytic effector activity in a 4-h ^{51}Cr-release assay against PHA blasts. Both autologous or stimulator cells were tested as target cells. Maximum and spontaneous release values were obtained by incubating targets with 1% Triton X-100 and medium alone, respectively. Triplicate assays were carried out at E/T ratios of 25:1, 50:1, and 100:1 in V-bottomed 96-well plates. Data are reported as mean percent specific ^{51}Cr-release.

RNA Blot Analysis. RNA was prepared from T cells (~1–3 $\times 10^7$/sample) by a rapid isolation procedure (31). RNA (10 μg) was fractionated on formaldehyde agarose gels, transferred and cross-linked to Zetaprobe membranes (Bio-Rad Laboratories, Cambridge, MA). Probes for IL-2, IL-4, IFN-γ and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) have been previously described (19, 32, 33). DNA fragments were purified and labeled with ^{32}P using a random priming kit (Boehringer Mannheim Corp., Indianapolis, IN). The prehybridized membranes were sequentially hybridized with different ^{32}P-labeled probes. Between hybridizations, each probe was stripped from the blots by boiling in a solution of SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS.

Results

Induction of Antigen-specific Hyporesponsiveness by Fab Fragments of Anti-CD28 mAb 9.3. Monovalent Fab fragments of the anti-CD28 mAb 9.3 can block T cell proliferative responses to alloantigen in primary MLC, by preventing the interaction of CD28 with its natural ligand B7/BB1 expressed on APCs (12). To evaluate whether there is a long-lasting effect of blocking the CD28 receptor during the initial exposure to antigen, we performed restimulation experiments. Lymphocytes were cultured with alloantigen for 7 d in separate flasks in medium containing 5 μg/ml Fab of 9.3 mAb or control mAb. Cells were then washed to remove mAb, cultured in fresh medium for an additional 3 d, and then restimulated with irradiated PBMC from either the original donor (Fig. 1, *left*) or from a third party donor (Fig. 1, *right*) in medium without mAb. Cells primed in the presence of control mAb and restimulated with PBMC originally used for priming showed a typical accelerated secondary proliferative response peaking on day 3. In contrast, those same primed cells showed a typical primary response, peaking on day 6, when stimulated with PBMC from a third party donor. Cells primed in the presence of 9.3 Fab, however, showed a decreased response when challenged with PBMC from the original donor, yet responded normally to PBMC from a third party donor. These results demonstrate that the secondary proliferative response of human T cells can be inhibited in

[1] *Abbreviations used in this paper:* CTLp, cytolytic precursor; MLC, mixed leukocyte culture.

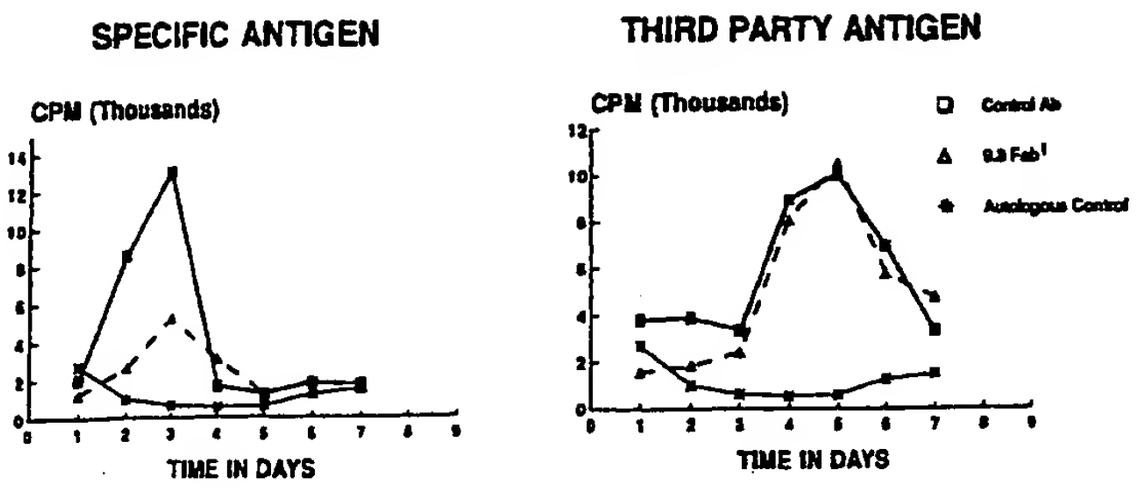


Figure 1. Fab fragments of anti-CD28 mAb 9.3 induce T cell hyporesponsiveness in MLC. For the secondary MLR, responders from a normal individual were primed with cells from an HLA-incompatible donor in the presence of 9.3 mAb Fab (*triangle*) or control (*square*). Cells were restimulated from the original donor (*left*) or a third party donor (*right*) in the absence of mAb. Cells primed in the absence of mAb were restimulated against autologous cells to define the background for the assay (*).

an antigen-specific manner by blocking CD28 during the primary exposure to alloantigen.

Inhibition of T Cell Responses to Alloantigen by CTLA4Ig. To determine whether antigen-specific hyporesponsiveness could also be induced by blocking B7/BB1, the natural ligand for CD28 expressed on APC, further experiments evaluated the activity of CTLA4Ig, a fusion protein with high affinity for B7/BB1. Both responder and irradiated stimulator cells were preincubated with CTLA4Ig or control Ig for 30 min at 37°C before mixing. CTLA4Ig inhibited primary alloproliferative responses by 50–85%, and maximal inhibition was seen at or above 2.5 μg/ml of CTLA4Ig (data not shown), consistent with previous findings (23). mAb BB1 (26), a murine IgM antibody that binds to the B7/BB1 antigen with lower avidity than CTLA4Ig (23) inhibited MLR by only ~30%. Thus, CTLA4Ig inhibited primary T cell responses more efficiently than mAb BB1, although the inhibition achieved was not complete.

Previous studies had shown that $CD4^+/CD28^+$ T cells constitute 95–99.5% of $CD4^+$ peripheral blood T cells and proliferate vigorously to HLA class II determinants in MLR (34), whereas $CD4^+/CD28^-$ T cells constitute 0.5–5.0% of all $CD4^+$ T cells and respond poorly in MLR (35), and $CD8^+$ T cells do not proliferate at all in human MLR. By flow microfluorimetric analysis we found that $CD4^+$ cells constituted 79% of viable lymphocytes on day 6 of an MLR carried out in the presence of control Ig compared with 56% in the presence of CTLA4Ig, and $CD28^+$ cells constituted 72% of viable lymphocytes after an MLR carried out in the presence of control Ig compared with 56% in the presence of CTLA4Ig. Thus, CTLA4Ig blocked the increase in the proportion of $CD4^+$ and $CD28^+$ cells during MLR. Since requirements for proliferation are more stringent in naive than in memory cells, one expected MLR response of naive cells to be more susceptible to inhibition by CTLA4Ig than MLR response of memory cells. $CD4^+/CD45RA^+$ (naive) and $CD4^+/CD45RO^+$ (memory) T cell subsets were purified by negative selection, through panning of PBMC obtained from adult volunteers, and tested in MLR. CTLA4Ig inhibited thymidine uptake of $CD4^+/CD45RA^+$ cells by 84% and $CD4^+/CD45RO^+$ cells by 74%. As an alternative source of naive T cells, mononuclear cells were obtained from umbilical cord blood and tested in MLR. CTLA4Ig inhibited thymidine uptake of cord blood cells stimulated by irradiated PBMC obtained from an unrelated adult by 78%. These results indicate that CTLA4Ig can inhibit proliferative responses to HLA class II determinants in either naive or memory T cells with the $CD4^+$ and $CD28^+$ phenotype. However, in no T cell subset analyzed was the inhibition complete.

Effect of CTLA4Ig on Lymphokine Production. Proliferative T cell responses to alloantigen occurring despite the presence of CTLA4Ig might not be driven by IL-2, but rather by IL-4. Steady state message for IL-2, IL-4, and IFN-γ was measured in mRNA prepared from proliferative $CD4^+$ T cell lines stimulated by specific alloantigen in the presence or absence of CTLA4Ig (Fig. 2). Transcripts for IL-2 and IFN-γ were lower in mRNA from cells cultured with CTLA4Ig compared with control cells. In contrast, transcripts for IL-4 peaked at 4 h after stimulation and were detected at similar levels in mRNA from cells cultured with or without CTLA4Ig. Thus, IL-2 and IFN-γ transcripts do not accumulate in T cells stimulated by alloantigen when B7/BB1 is blocked by CTLA4Ig, whereas IL-4 transcripts do accumulate. Therefore, IL-4 could drive antigen-specific T cell proliferation which occurs despite blocking by CTLA4Ig.

Induction of Antigen-specific Hyporesponsiveness by CTLA4Ig. To evaluate the effect of CTLA4Ig on secondary responses,

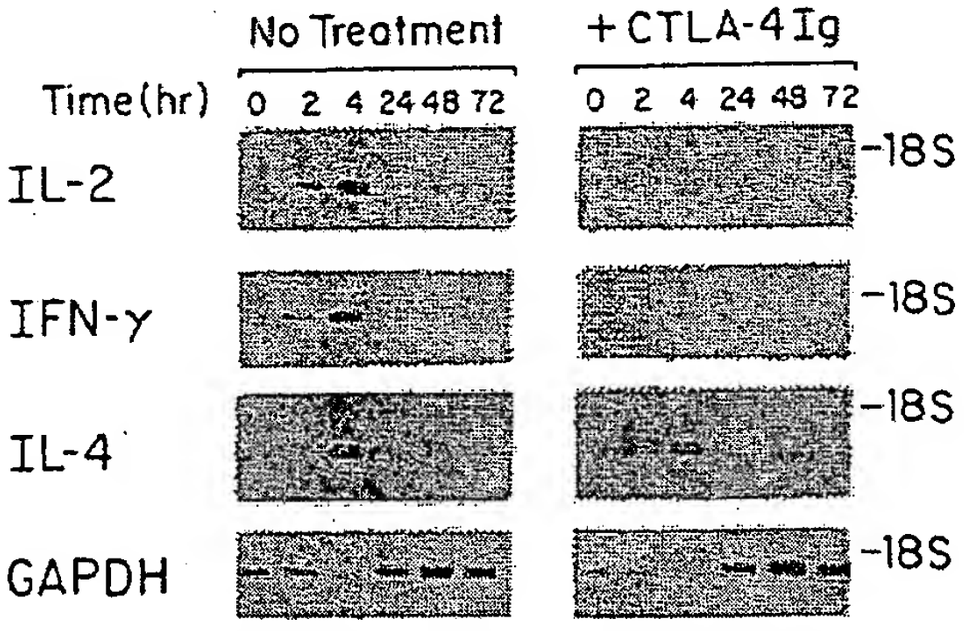


Figure 2. Regulation of lymphokine transcripts by CTLA4Ig. Resting alloantigen-primed $CD4^+$ T cells (2×10^7) were collected and restimulated with irradiated lymphoblastoid cells. Cells were harvested at the indicated times, RNA was extracted and analyzed by blot analysis. The blot was sequentially hybridized with ^{32}P-labeled probes for IL-2, IL-4, IFN-γ and GAPDH, as described in Material and Methods. Migration positions are noted of the 28S and 18S ribosomal RNA species visualized by ethidium bromide staining.

lymphocytes were cultured with alloantigen for 7 d in medium containing 5 μg/ml CTLA4Ig or control Ig. Cells were then washed to remove Ig, cultured in fresh medium for an additional 3 d, and then restimulated with irradiated PBMC from either the original donor (Fig. 3, *top left*) or from a third party donor (Fig. 3, *bottom left*) in medium without Ig. Cells primed in the presence of CTLA4Ig showed a decreased response when challenged with PBMC from the original donor, yet responded normally to PBMC from a third party donor. Flow microfluorimetric analysis of $CD4^+$ cells on day 3 of the secondary cultures indicated that expression of the IL-2 receptor α chain (CD25) was lower in CTLA4Ig-treated cultures than in controls (data not shown). Antigen-specific hyporesponsiveness was achieved with as low as 1 μg/ml of CTLA4Ig in the priming culture, but there was no effect on responsiveness to third party donors even at a CTLA4Ig concentration of 10 μg/ml (data not shown). Hyporesponsiveness was demonstrated in cells cultured with alloantigen in the presence of CTLA4Ig for 7 d, and then rested in medium alone for 20 d and 27 d after initiation of the culture (Fig. 3). In six experiments using different pairs of responder and stimulator cells, primary MLC in the presence of CTLA4Ig inhibited the secondary proliferative responses to the specific alloantigens by an average (±SD) of 70 ± 13%, whereas responses to third party donors were unaffected (4 ± 3% inhibition). Secondary proliferative responses to specific alloantigen were inhibited by an identical degree, if the primary cultures were carried out in the presence of either CTLA4Ig (84% inhibition) or anti-CD28 mAb Fab (83% inhibition), but no greater inhibition was achieved by a combination of the two (84% inhibition). These results demonstrate that secondary proliferative responses can be specifically inhibited by primary exposure of T cells to alloantigen in the presence of either anti-CD28 mAb Fab fragments or CTLA4Ig, and are consistent with the model that both agents block the same pathway of T cell activation.

Effect of CTLA4Ig on Responsiveness of Primed Cells. Further experiments were designed to determine whether alloantigen-specific hyporesponsiveness could be induced by CTLA4Ig in primed cells. Cells were primed to alloantigen in medium without CTLA4Ig or control for 10 d (Fig. 4, *top left*). Cells were then washed and restimulated with irradiated cells from the original donor. Both responder and stimulator cells were incubated with CTLA4Ig or control Ig for 30 min at 37°C before mixing. The secondary proliferative response was inhibited by CTLA4Ig compared with the Ig control (Fig. 4, *top right*). In separate cultures set up in flasks, primed cells were restimulated with PBMC from the original donor in the presence of CTLA4Ig or control Ig for 7 d, washed to remove the Ig, and rested in medium for

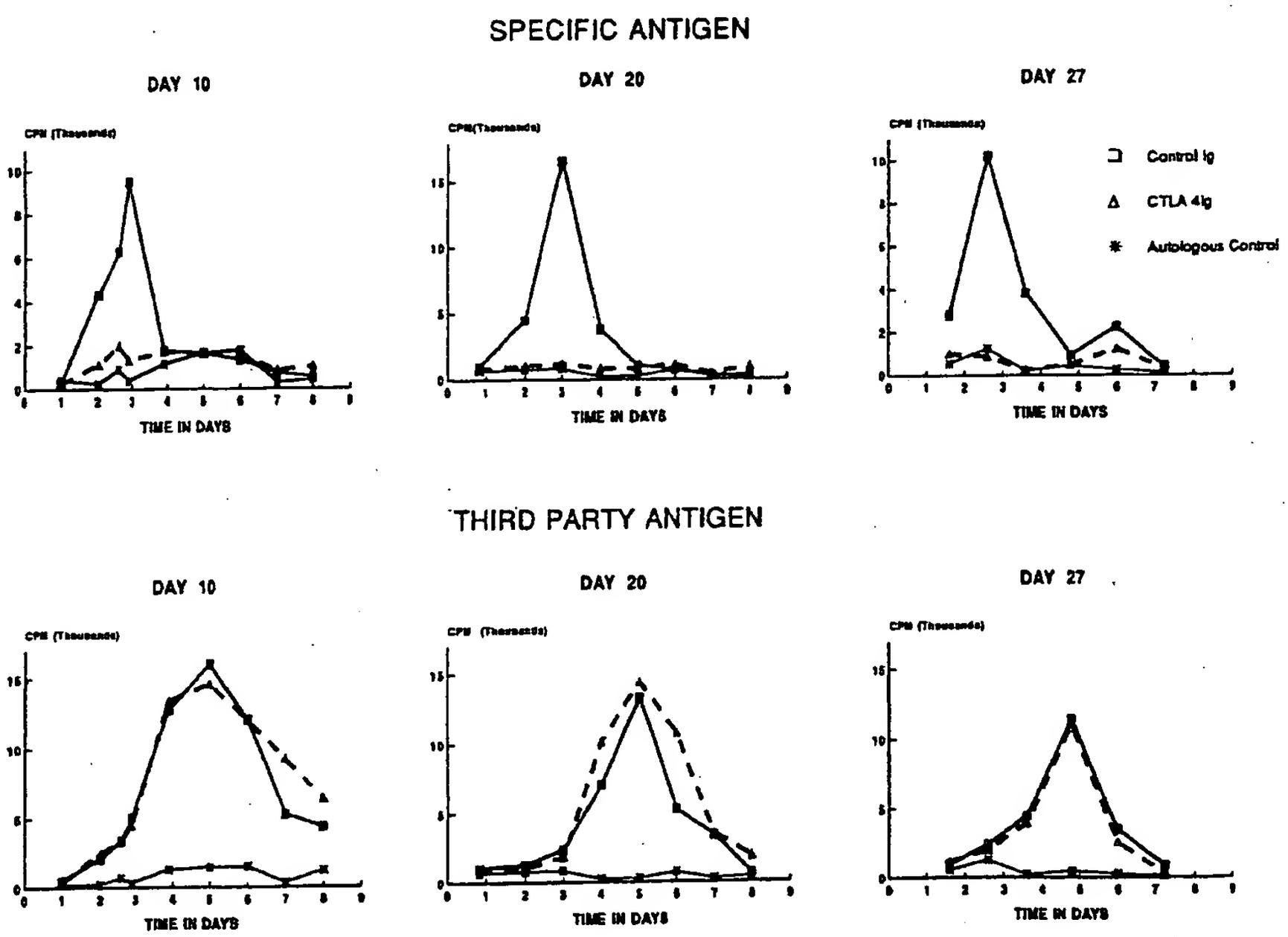


Figure 3. CTLA4Ig induces antigen-specific hyporesponsiveness in unprimed cells. Responders from a normal individual were primed with cells from an HLA-incompatible donor in the presence of CTLA4Ig (*triangle*) or control Ig (*square*). At the indicated time points, primed cells were restimulated from the original donor (*top*) or a third party donor (*bottom*) in the absence of Ig. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (*).

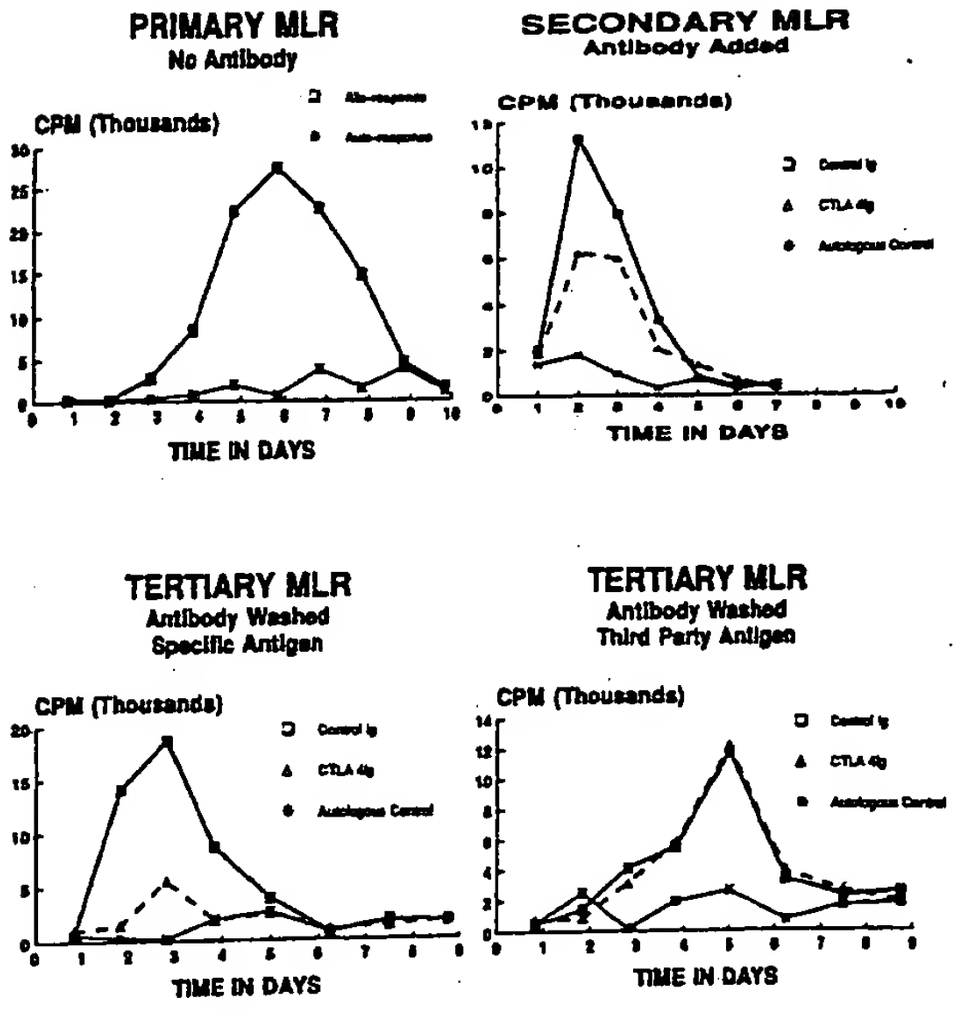


Figure 4. CTLA4Ig induces antigen-specific hyporesponsiveness in primed cells. PBMC were primed with allogeneic stimulators (*square*) in absence of Ig (*top left*; [•] autologous stimulation). Primed cells were restimulated in secondary MLR with cells from the original donor (*top right*) in the presence of CTLA4Ig (*triangle*) or control Ig (*square*). Cells from the respective cultures were washed and then restimulated in a tertiary MLR (*bottom*) with cells from the original donor (*left*) or a third party donor (*right*) in absence of Ig. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (•).

an additional 3 d. They were then stimulated again in a tertiary culture with specific or third party alloantigen. Cells preincubated with control Ig exhibit a typical anamnestic response when restimulated with cells from the original donor. In contrast, cells preincubated with CTLA4Ig showed a diminished response to cells from the original donor (Fig. 4, *bottom left*), whereas their response to a third party donor was unaffected (Fig. 4, *bottom right*). These findings indicate that primed cells can also become hyporesponsive if exposed to alloantigen in the presence of CTLA4Ig.

Kinetics of Induction of Hyporesponsiveness by CTLA4Ig. To determine the duration of exposure to CTLA4Ig necessary for development of hyporesponsiveness, cells were washed on days 1, 2, or 3 of primary MLC, resuspended in fresh medium without Ig, and rested until day 10 when they were restimulated with irradiated PBMC from the original donor or from a third party donor. Primary MLC in the presence of CTLA4Ig for 2 or 3 d inhibited the secondary response to the original donor >80%, but had no effect on the response to third party donors. Primary MLC in the presence of CTLA4Ig for 1 d inhibited the secondary response to the original donor by only ~15%. Therefore, maximum induction of antigen-specific hyporesponsiveness is achieved in MLC after 2 d of incubation with CTLA4Ig.

Effect of IL-2 on Hyporesponsive Cells. Hyporesponsiveness in secondary MLR could be due to the death of antigen-specific T cells occurring during the primary culture or to the acquisition of a defect in one of the cellular functions that limits the rate of cell proliferation, such as IL-2 production. Addition of exogenous IL-2 to secondary cultures could help determine whether IL-2-responsive, antigen-specific T cells were still alive. Primary MLCs were set up in medium containing CTLA4Ig or control Ig. When challenged with PBMC from the original donor, cells primed in the presence of CTLA4Ig showed a lower response (Fig. 5, *center*) than cells primed in the presence of control Ig (Fig. 5, *left*), yet responded equally well to PBMC from a third party donor. Exogenous rIL-2 added at 10 IU/ml to secondary cultures restored responsiveness to specific alloantigen of cells primed in the presence of CTLA4Ig (Fig. 5, *right*). These results indicate that presentation of antigen while blocking interaction of CD28 with B7/BB1 can induce a state of T cell hyporesponsiveness to antigen which can be corrected by exogenous IL-2.

Effect of IL-2 on Induction of Antigen-specific Hyporesponsiveness by CTLA4Ig. CD28 signaling concurrent with TCR engagement results in IL-2 secretion, T cell activation and proliferation. Therefore, we tested whether exogenous IL-2 could provide T cells with a signal that could bypass the block provided by CTLA4Ig in the primary MLC and prevent induction of antigen-specific hyporesponsiveness. Primary MLCs were set up with CTLA4Ig, with or without rIL-2 at 10 IU/ml

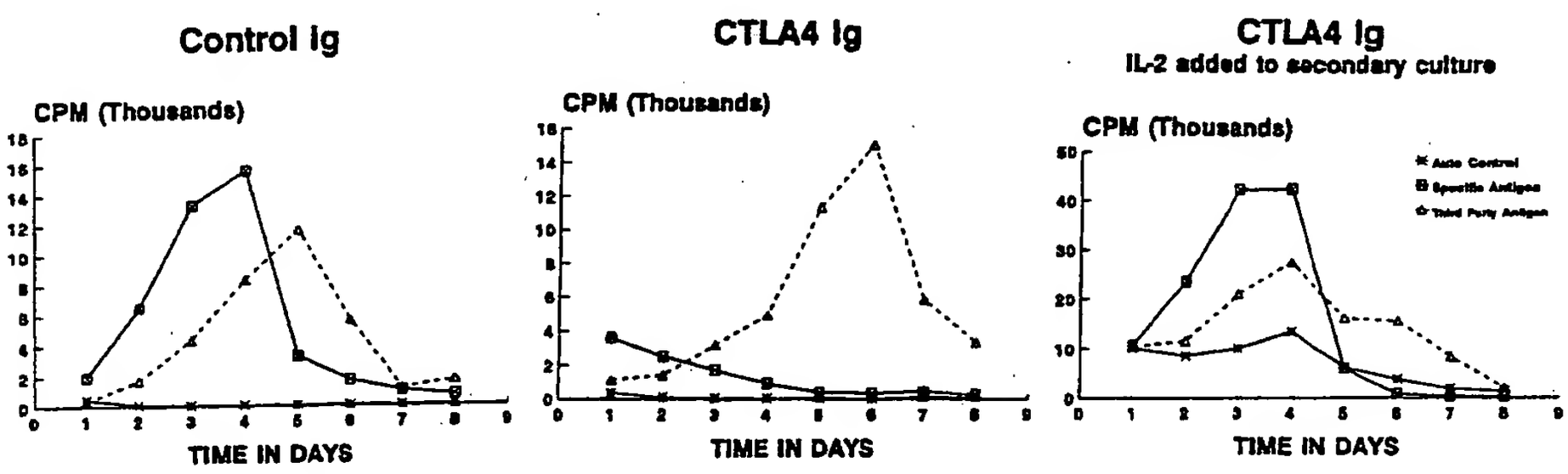


Figure 5. Exogenous IL-2 restore responsiveness to specific antigen. Cells were primed to alloantigen in the presence of control Ig (*left*) or CTLA4Ig (*center* and *right*). Primed cells were restimulated with autologous cells (•), cells from the original donor (*square*) or a third party donor (*triangle*) in the absence of Ig. Cells primed in the presence of CTLA4Ig were restimulated in medium (*center*) or 10 μ rIL-2 (*right*).

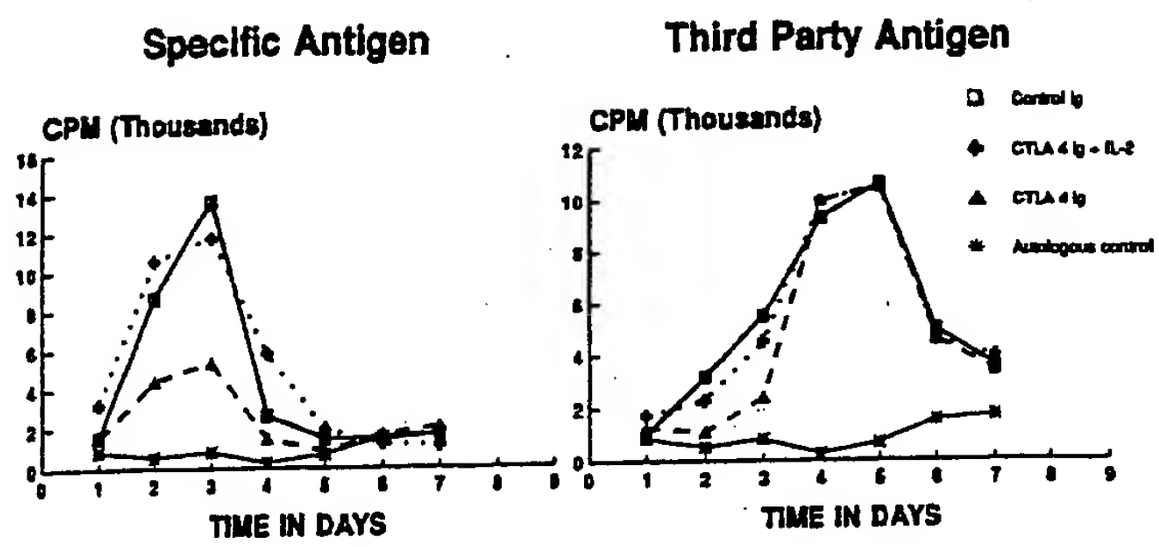


Figure 6. Exogenous IL-2 prevents induction of antigen-specific hyporesponsiveness by CTLA4Ig. Cells were primed in the presence of CTLA4Ig (*triangle*), or control Ig (*square*), or CTLA4Ig plus IL-2 (*cross*). Primed cells were restimulated with cells form the original donor (*left*) or a third party donor (*right*) in the absence of Ig or IL-2. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (*).

added at the initiation of the culture or control Ig. Cells primed in the presence of CTLA4Ig alone showed hyporesponsiveness when restimulated with the specific alloantigen (Fig. 6, *left*). However, cells primed in the presence of CTLA4Ig plus rIL-2 showed the same degree of secondary response to the specific stimulators as cells primed in the presence of control Ig. Neither CTLA4Ig nor rIL-2 affected secondary responses to cells from third party donors (Fig. 6, *right*). These results indicate that antigen-specific hyporesponsiveness induced by priming T cells in the presence of CTLA4Ig in MLC can be prevented by stimulation with exogenous IL-2.

Effect of Anti-CD28 mAb 9.3 or Cell-bound B7/BB1 Receptor on Induction of Antigen-specific Hyporesponsiveness by CTLA4Ig. In contrast to monovalent Fab fragments of anti-CD28 mAb 9.3, the bivalent intact mAb 9.3 can crosslink CD28 molecules and activate T cells efficiently (29). Therefore, we tested whether the intact mAb 9.3 could deliver a signal to T cells and prevent induction of hyporesponsiveness by CTLA4Ig. Primary MLCs were set up with CTLA4Ig with or without mAb 9.3 or controls. Cells primed in the presence of CTLA4Ig alone showed hyporesponsiveness, compared with cells primed in the presence of control Ig alone (not shown) or control Ig plus mAb 9.3, when restimulated with the specific alloantigen (Fig. 7, *left*). However, cells primed in the presence of CTLA4Ig plus mAb 9.3 showed the same degree of secondary response to the specific stimulators as was shown by cells primed in the presence of control Ig and mAb 9.3. Neither CTLA4Ig nor mAb 9.3 affected secondary responses to cells from third party donors (Fig. 7, *right*).

Further experiments tested the effect of exogenous B7/BB1 antigen expressed on transfected CHO cells. Irradiated (10^4 rad) $B7^+$ CHO cells (19) were mixed with fresh responder PBMC at a ratio of 1:100, before addition of CTLA4Ig or control Ig and irradiated stimulator PBMC. MLC without CHO cells but with CTLA4Ig or control Ig alone were set up in parallel. Cells primed in the presence of CTLA4Ig alone showed hyporesponsiveness to specific alloantigen when compared with cells primed in the presence of control Ig. Cells primed in the presence of CTLA4Ig and the negative control $CD5^+$ CHO cells also showed hyporesponsiveness. In contrast, cells primed in the presence of CTLA4Ig and $B7^+$ CHO cells showed the same degree of secondary response to the specific stimulators, as was shown by cells primed in the presence of control Ig and no CTLA4Ig (data not shown). Neither CTLA4Ig nor transfected CHO cells affected secondary responses to cells from third party donors (data not shown). These results indicate that antigen-specific hyporesponsiveness induced by priming T cells in the presence of CTLA4Ig in MLC can be prevented by stimulating CD28 with mAb 9.3 or with the natural ligand B7/BB1.

Effect of CTLA4Ig on CTL Generation. CTLA4Ig did not inhibit the effector phase of the cytolytic reaction by activated CTL against allogeneic target T cell blasts (data not shown). To determine whether CTLA4Ig added to the primary MLC could block the generation of CTL activity, MLCs were set up in medium containing CTLA4Ig or control Ig for 5 d. Cells primed in the presence of CTLA4Ig showed a fourfold decrease in cytolytic activity against allogeneic target T cell blasts when compared with cells primed in the presence of control Ig (data not shown). To determine whether the block in the generation of CTL activity by CTLA4Ig was specific, MLCs were set up in medium containing

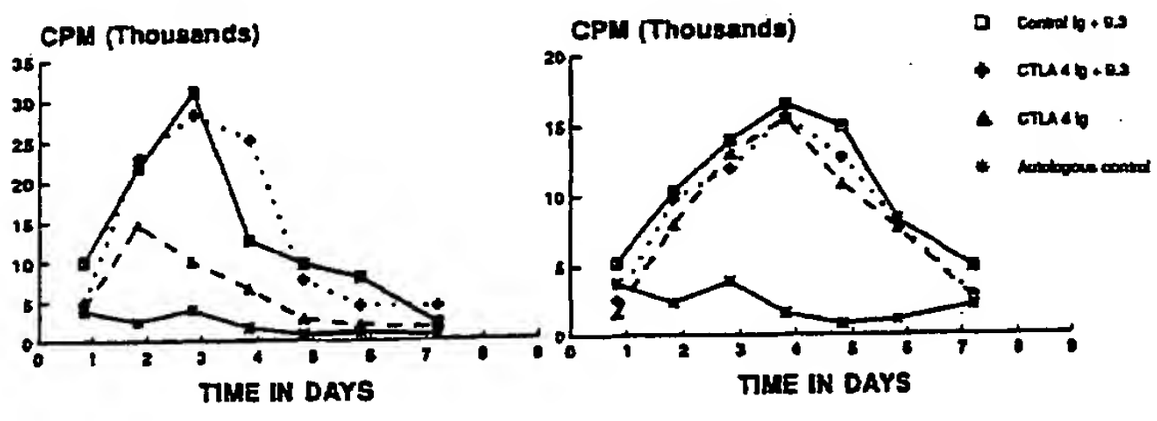


Figure 7. Intact mAb 9.3 blocks induction of antigen-specific hyporesponsiveness by CTLA4Ig. Cells were primed in the presence of CTLA4Ig (*triangle*), or control Ig plus mAb 9.3 (*square*), or CTLA4Ig plus mAb 9.3 (*cross*). Primed lymphocytes were restimulated with cells from the original donor (*left*) or a third party donor (*right*) in the absence of Ig. Cells primed in the absence of Ig were restimulated against autologous cells to define the background for the assay (*). This experiment is representative of two other experiments of similar design that achieved identical results.

CTLA4Ig or control Ig for 7 d. Cells were washed and recultured in fresh medium without CTLA4Ig for 3 d. Cells were then restimulated with irradiated PBMC from the original donor or from a third party donor for 3 d and then tested for cytolytic activity. Cells previously primed in the presence of CTLA4Ig again showed a fourfold decrease in cytolytic activity against specific alloantigen (Fig. 8, *left*) when compared with cells primed in the presence of control Ig. In contrast, cells previously cultured in the presence of CTLA4Ig were able to generate cytotoxic activity against a third party donor to the same degree as cells cultured with control Ig (Fig. 8, *right*). Thus, CTLA4Ig inhibited the generation of specific CTL.

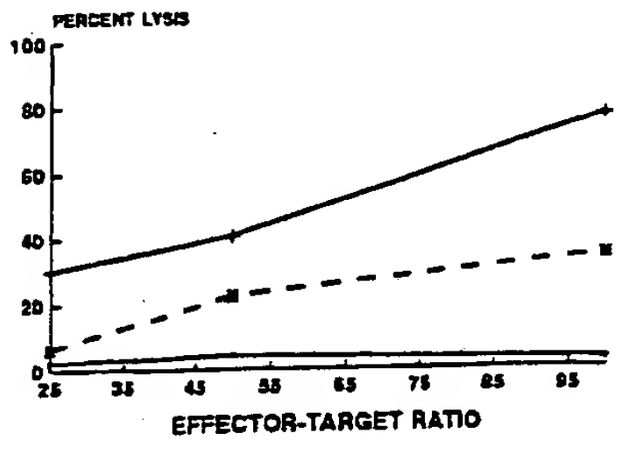
Discussion

Our study demonstrates that long-lasting, antigen-specific hyporesponsiveness can be induced in T cells by exposure to alloantigen while blocking the interaction of CD28 on T cells with B7/BB1 on allogeneic APC. Effective blockade could be achieved using either monovalent anti-CD28 mAb 9.3 fragments or CTLA4Ig, a soluble recombinant fusion product of human CTLA-4 and IgG γ1 chain, the binds to B7/BB1 with high avidity. Previous reports had shown that anti-CD28 mAbs augment proliferation of human T cells in the presence of specific antigen and defective APCs (16). Further work showed that interaction of CD28 with B7/BB1 provides a costimulatory signal for T cell activation (17–20). Data from Schwartz and other investigators (4–7, 36) indicated that in the absence of costimulatory signals provided by the APC, T cells encountering specific antigen enter a state of anergy characterized by an IL-2 production defect. Harding et al. (21) demonstrated that CD28 signaling can prevent anergy in murine T cell clones. Our data are consistent with the model that the CD28–B7/BB1 interaction can provide a costimulus required for T cell activation. CD28 ligation is required for IL-2 gene activation in antigen-specific responses. Blocking CD28 ligation by either 9.3 mAb Fab or CTLA4Ig may inhibit IL-2 expression and elicit a state of T cell hyporesponsiveness.

Human MLR experiments allowed us to study the requirements for antigen-specific responses of $CD4^+/CD28^+$ cells, since proliferation of $CD4^+/CD28^-$ cells and $CD8^+$ cells cannot be detected in this model system. CTLA4Ig blocked proliferation of $CD4^+/CD28^+$ in primary MLC and achieved a similar degree of inhibition in naive and memory T cells. A state of antigen-specific hyporesponsiveness could be induced by CTLA4Ig in primed as well as in unprimed cells. Secondary proliferative responses to the specific alloantigen were decreased, but not abolished by the presence of either anti-CD28 mAb 9.3 Fab or CTLA4Ig in primary cultures. Residual T cell responsiveness hardly could be explained by incomplete blocking of the CD28 or B7/BB1 receptors, since anti-CD28 mAb Fab and CTLA4Ig used together did not demonstrate additive inhibitory activity. T cell proliferation could be induced through CD28-independent activation pathways initiated by other accessory receptors, such as ICAM-1 (10). The heat-stable antigen also functions as a costimulatory molecule and regulates T cell responsiveness (37). CTLA4Ig blocked antigen-mediated activation of IL-2 and IFN-γ expression, but did not block IL-4 expression. Thus, IL-4 might be responsible for driving T cell proliferation in primary or secondary MLR. Further experiments will need to address whether neutralization of IL-4 in culture can achieve complete T cell unresponsiveness in this model.

Exogenous IL-2 could restore antigen-specific proliferative responses in secondary cultures, suggesting that hyporesponsiveness was not the result of T cell death but, instead, was likely the result of an acquired T cell defect in IL-2 production. Whether blocking alloantigen-mediated T cell activation by CTLA4Ig can induce a sustained defect in IL-2 production remains to be verified. Exogenous IL-2 added at the beginning of the primary MLC prevented induction of antigen-specific hyporesponsiveness by CTLA4Ig in this study. This finding contrasts with the observation in the model using murine T cell clones stimulated in absence of accessory cells, where exogenous IL-2 cannot prevent hyporesponsiveness. As opposed to the murine model, MLR cultures contain accessory cells and with them an indefinite number of stimuli that could make less stringent the requirements for T cell activation.

Both CD28 and CTLA-4 are natural ligands for B7/BB1, a receptor expressed on activated B lymphocytes and other APCs (38–41). Once expressed, B7/BB1 interacts with CD28 and CTLA-4 to provide a stimulus for T cell activation (18, 42). The time required for B7 expression, 16–24 h after B cell activation (39), can explain why T cell hyporesponsive-



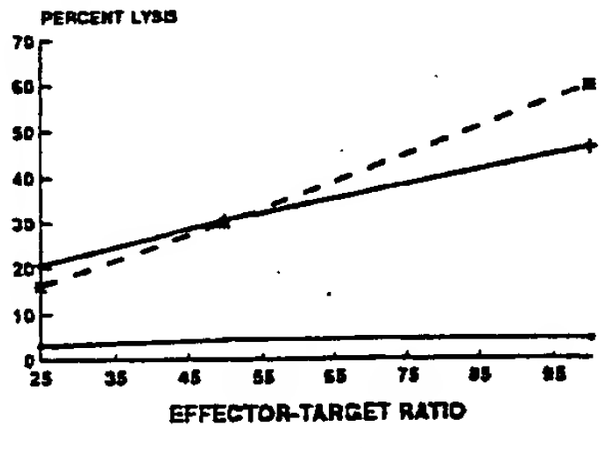


Figure 8. CTLA4Ig inhibits CTL generation. Responder cells were primed in the presence of CTLA4Ig (*) or control Ig (*cross*). Primed cells were restimulated with cells from the original donor (*left*) or from a third party donor (*right*) in the absence of Ig and cytotoxic activity was assayed on day 3 of the secondary cultures against PHA stimulated T lymphoblasts from the respective donors. Lysis of autologous targets (*square*) by cells primed in absence of Ig define the background for the assay. This experiment is representative of two other experiments of similar design that achieved identical results.

ness is not completely achieved in MLC by 24 h but requires 48 h of incubation with CTLA4Ig. CD28 ligation by the whole anti-CD28 mAb 9.3 could prevent induction of hyporesponsiveness by CTLA4Ig in MLC. Hyporesponsiveness was also prevented by mixing irradiated B7+ CHO cells with responder lymphocytes at the initiation of the MLC before adding CTLA4Ig. Prevention of hyporesponsiveness by B7+ CHO cells may be achieved by direct stimulation of T cells through CD28 or by neutralization of soluble CTLA4Ig. We favor the former hypothesis since B7+CHO cells were used at a very low frequency in the culture (1:100:100, B7+ CHO cells/responders/stimulators). Under these experimental conditions, we calculated that the concentration of CTLA4Ig in the culture exceeded the concentration of the B7/BB1 receptor on the surface of CHO cells by at least 100-fold on a molar basis. Thus, it was unlikely that CTLA4Ig could be neutralized by B7+ CHO cells. The role of the CTLA-4 receptor in the achievement of T cell hyporesponsiveness has not been addressed directly in our studies. However, since blocking CD28 by 9.3 mAb Fabs induced a level of hyporesponsiveness comparable to blocking B7/BB1 by CTLA4Ig, and since triggering CD28 by the intact 9.3 mAb could completely overcome hyporesponsiveness induced by CTLA4Ig, it is unlikely that signaling by CTLA-4 per se is of major importance in regulating T cell responses.

CTLA4Ig not only blocked primary and secondary proliferative responses but also blocked activation of cytolytic precursors (CTLp). Cells exposed to alloantigen in the presence of CTLA4Ig were found to generate markedly diminished specific cytolytic activity. These results suggest that generation of cytolytic activity in the primary culture in the presence of CTLA4Ig was an unlikely explanation for the hyporesponsiveness in secondary culture. Recent findings indicate that CD28 interaction with B7/BB1 can amplify T cell–mediated cytolysis at the effector phase (43). In our experiments, however, there was no interference of CTLA4Ig at the lytic stage, probably because activated T cell targets do not express B7/BB1. It remains to be determined whether CTLA4Ig blocked CTLp activation directly or indirectly by inhibiting Th cell functions.

Defining the role of the interaction between CD28 and B7/BB1 and between other T cell accessory receptors and their natural ligands will help understand the mechanisms for self-tolerance, propose new strategies to manipulate the immune response, and achieve transplantation tolerance (24, 25, 44).

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